



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) **EP 1 317 537 B1**

(12)

EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention
of the grant of the patent:
20.12.2006 Bulletin 2006/51

(21) Application number: **01970897.3**

(22) Date of filing: **10.09.2001**

(51) Int Cl.:
C12N 15/00 (2006.01)

(86) International application number:
PCT/US2001/028602

(87) International publication number:
WO 2002/020766 (14.03.2002 Gazette 2002/11)

(54) G-CSF ANALOG COMPOSITIONS AND METHODS

ZUSAMMENSETZUNGEN UND VERFAHREN MIT ANALOGEN VON G-CSF

COMPOSITIONS D'ANALOGUES DE G-CSF ET PROCEDES CORRESPONDANTS

(84) Designated Contracting States:
**AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE TR**

(30) Priority: **08.09.2000 US 231464 P**

(43) Date of publication of application:
11.06.2003 Bulletin 2003/24

(73) Proprietor: **MASSACHUSETTS INSTITUTE OF
TECHNOLOGY**
Cambridge, MA 02139 (US)

(72) Inventors:
• **SARKAR, Casim, A.**
Cambridge, MA 02139 (US)

• **LAUFFENBURGER, Douglas, A.**
Cambridge, MA 02139 (US)
• **TIDOR, Bruce**
Lexington, MA 02421-4115 (US)

(74) Representative: **Grünecker, Kinkeldey,
Stockmair & Schwanhäusser**
Anwaltssozietät
Maximilianstrasse 58
80538 München (DE)

(56) References cited:
EP-A- 0 456 200 **EP-A- 0 459 630**
US-A- 4 810 643

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

Description

FIELD OF THE INVENTION

5 **[0001]** The present invention relates to granulocyte colony stimulating factor ("G-CSF") polypeptide analog compositions, related nucleic acids, and vectors, host cells and processes for recombinant DNA production of the present G-CSF analog polypeptides. In addition, pharmaceutical compositions, and methods of use are provided. Some aspects of the invention should be generalizable beyond G-CSF analog compositions as well.

10 BACKGROUND OF THE INVENTION

[0002] Many therapeutic ligands elicit cellular responses by binding to cell-surface receptors to elicit cellular responses. Drug design is typically focused on the ability of a ligand to bind tightly and specifically to its intended target. However, if the drug is a protein and the target a cell-surface receptor, there are additional issues to consider from a systems-level analysis. When therapeutic ligands bind to receptors on the surface of a cell, an intracellular signaling cascade is initiated that ultimately results in an appropriate cellular response. Additionally, modulation - generally attenuation - of these signals begins almost immediately by cellular trafficking of the ligand-receptor complexes. The complexes on the surface of the cell are internalized into vesicles that fuse with endosomal compartments. From endosomes, the molecules can either be routed to degradation in lysosomes or be recycled to the cell surface intact, where free and ligand-bound receptor are redisplayed and free ligand is released to the extracellular medium. Recent evidence suggests the outcome of this sorting decision for complexes involving growth factors or cytokines often is related to the endosomal affinity constant for the ligand-receptor interaction: complexes that remain bound are readily degraded while those that dissociate are recycled [Lauffenburger *et al.*, *Chem. Biol.* 5:R257-R263 (1998)]. In general, dissociation of complexes in endosomes appears to enhance receptor recycling, because it results in altered interactions between the receptors and endosomal retention components.

[0003] Additionally, for a low number of intracellular complexes, which is the case for many clinically important cytokine-receptor systems, modeling indicates a particularly strong positive correlation between the inverse endosomal affinity and the fraction of ligand recycled [French and Lauffenburger, *Ann. Biomed. Eng.* 25:690-707 (1997)]. Thus, if a ligand could be designed to enhance endosomal dissociation after binding to and generating signals within its target cell, the drug might reduce receptor downregulation, so that cells would be more responsive to further ligand stimulation. The lifetime and effectiveness of the drug might also be enhanced if ligand recycling were augmented by endosomal dissociation. This contrasts with the conventional approach of attempting to improve ligand potency through enhanced affinity. If extracellular affinity enhancements extend to endosomes, such attempts might actually be counterproductive because they increase receptor downregulation and possibly ligand depletion. Thus, cellular trafficking may be a bottleneck in enhancing ligand potency, particularly in cases where degradation through receptor-mediated endocytosis is significant.

[0004] A system in which the optimization of cellular trafficking properties could have a profound impact on potency is that of granulocyte colony-stimulating factor (G-CSF) and its receptor (G-CSFR). G-CSF is a 19-kDa cytokine, which is one of the hematopoietic growth factors, also called colony stimulating factors. G-CSF is used to increase white blood cell (neutrophil) counts when blood levels of such cells are dangerously low. This commonly occurs when certain antibiotics, anti-HIV therapies and/or chemotherapies suppress the bone marrow. A recent study documents that G-CSF not only increases the number of neutrophils in the blood, but enhances the functional killing abilities of those cells as well [Vecchiarelli *et al.*, *J. Infect. Dis.* 171:1448-1454 (1995)]. G-CSF specifically stimulates the proliferation and differentiation of neutrophilic precursor cells into mature neutrophils [Fukunaga *et al.*, *Cell* 74:1079-1087 (1993)], and is useful for treating in neutropenic states [Welte *et al.*, *Proc. Natl. Acad. Sci. USA* 82:1526-1530 (1985); Souza *et al.*, *Science* 232:61-65 (1986); Gabrilove, *Sem. Hematol.* 26(2):1-14 (1989)]. G-CSF increases the number of circulating granulocytes and has been reported to ameliorate infection in sepsis models. G-CSF administration also inhibits the release of tumor necrosis factor (TNF), a cytokine important to tissue injury during sepsis and rejection [Wendel *et al.*, *J. Immunol.* 149: 918-924 (1992)]. G-CSF is a member of the Group I superfamily of cytokines, characterized by an antiparallel 4-helical bundle structure and including other therapeutically important drugs such as erythropoietin and growth hormone. G-CSF binds specifically and with high affinity (apparent $K_D \sim 100$ pM) [Morstyn, Dexter, & Foote (eds.) *Filgrastim (r-metHuG-CSF)* in: *Clinical Practice*, Edn. 2., Marcel Dekker, Inc., New York (1998)] to G-CSFR, resulting in a ligand:receptor complex with a 2:2 stoichiometry [Horan *et al.*, *Biochemistry* 35:4886-4896 (1996); Horan *et al.*, *J. Biochem.* 121:370-375 (1997)]. The extracellular region of G-CSFR contains the ligand-binding cytokine receptor homology (CRH) domain [Fukunaga *et al.*, *EMBO J.* 10:2855-2865 (1991)] and recently, the crystal structure of G-CSF complexed with the CRH domain of G-CSFR was solved, showing the expected 2:2 ligand:receptor stoichiometry [Aritomi *et al.*, *Nature*, 401: 713-717 (1999)].

[0005] In humans, endogenous G-CSF is detectable in blood plasma [Jones *et al.*, *Bailliere's Clin. Hematol.* 2(1): 83-111 (1989)]. G-CSF is produced by fibroblasts, macrophages, T cells, trophoblasts, endothelial cells, and epithelial

cells, and is the expression product of a single copy gene comprised of four exons and five introns located on chromosome seventeen. Transcription of this locus produces a mRNA species which is differentially processed, resulting in two forms of G-CSF mRNA, one version coding for a protein of 177 amino acids, the other coding for a protein of 174 amino acids [Nagata *et al.*, *EMBO J.* 5:575-581 (1986)]. The form comprised of 174 amino acids has been found to have specific *in vivo* biological activity. SEQ ID NO: 1 presents a DNA encoding the 174 amino acid species of G-CSF and the corresponding sequence of amino acids is set out in SEQ ID NO: 2. G-CSF is species cross-reactive, such that when human G-CSF is administered to another mammal such as a mouse, canine, or monkey, sustained neutrophil leukocytosis is elicited [Moore *et al.*, *Proc. Natl. Acad. Sci. USA* 84:7134-7138 (1987)].

[0006] Human G-CSF can be obtained and purified from a number of sources. Natural human G-CSF can be isolated from the supernatants of cultured human tumor cell lines. The development of recombinant DNA technology has enabled the production of commercial scale quantities of G-CSF in glycosylated form as a product of eukaryotic host cell expression, and of G-CSF in non-glycosylated form as a product of prokaryotic host cell expression. See, for example, US Patent No. 4,810,643 (Souza).

[0007] G-CSF has been found to be useful in the treatment of indications where an increase in neutrophils will provide benefits. For example, for cancer patients, G-CSF is beneficial as a means of selectively stimulating neutrophil production to compensate for hematopoietic deficits resulting from chemotherapy or radiation therapy. Other indications include treatment of various infectious diseases and related conditions, such as sepsis, which is typically caused by a metabolite of bacteria. G-CSF is also useful alone, or in combination with other compounds, such as other cytokines, for growth or expansion of cells in culture (for example, for bone marrow transplants or *ex vivo* expansion). G-CSF has been administered to transplant patients as an adjunct to treatment of infection or for treatment of neutropenia [Diflo *et al.*, *Hepatology* 16:PA278 (1992); Wright *et al.*, *Hepatology* 14:PA48 (1991); Lachaux *et al.*, *J. Ped.* 123:1005-1008 (1993); Colquehoun *et al.*, *Transplantation* 56:755-758 (1993)]. However, G-CSF is rapidly cleared through receptor-mediated endocytosis by peripheral neutrophils and precursor cells in bone marrow expressing G-CSFR [Morstyn, Dexter, & Foote (eds.) *Filgrastim (r-methHuG-CSF) in: Clinical Practice*, Edn. 2., Marcel Dekker, Inc., New York (1998)]. Thus, the potency of the drug is reduced by this negative feedback mechanism. Since cells naturally express G-CSFR in low numbers, decreasing the endosomal affinity of the complex may not only reduce receptor downregulation but may also enhance ligand recycling, as predicted by modeling [French and Lauffenburger, *Ann. Biomed. Eng.* 25:690-707 (1997)]. Therefore, G-CSF is a prime candidate for mutagenesis to enhance trafficking properties, thereby improving drug potency.

[0008] Various altered G-CSF's have been reported. Generally, for design of drugs, certain changes are known to have certain structural effects. For example, deleting one cysteine could result in the unfolding of a molecule which, in its unaltered state, is normally folded via a disulfide bridge. There are other known methods to one skilled in the art for adding, deleting or substituting amino acids in order to change the function of a protein.

[0009] Recombinant human G-CSF mutants have been prepared, but the method of preparation does not include overall structure/function relationship information. For example, the mutation and biochemical modification of Cys 18 has been reported [Kuga *et al.*, *Biochem. Biophys. Res. Comm.* 159:103-111 (1989); Lu *et al.*, *Arch. Biochem. Biophys.* 268:81-92 (1989)].

[0010] In US Patent No. 4,810,643, entitled, "Production of Pluripotent Granulocyte Colony-Stimulating Factor" (incorporated by reference herein), polypeptide analogs and peptide fragments of G-CSF are disclosed generally. Specific G-CSF analogs disclosed include those with the cysteines at positions 17, 36, 42, 64, and 74 (of the 174 amino acid species or of those having 175 amino acids, the additional amino acid being an N-terminal methionine) substituted with another amino acid (such as serine), and G-CSF with an alanine in the first (N-terminal) position.

[0011] EP 0 335 423 entitled "Modified human G-CSF" reportedly discloses the modification of at least one amino group in a polypeptide having hG-CSF activity.

[0012] EP 0 272 703 entitled "Novel Polypeptide" reportedly discloses G-CSF derivatives having an amino acid substituted or deleted at or "in the neighborhood" of the N-terminus. Also, Okabe *et al.* [Blood 75(9):1788-1793 (1990)], reportedly discloses modifications of five positions of the N-terminal region of human G-CSF.

[0013] EP 0 459 630, entitled "Polypeptides" reportedly discloses derivatives of naturally occurring G-CSF having at least one of the biological properties of naturally occurring G-CSF and a solution stability of at least 35% at 5 mg/mL in which the derivative has at least Cys¹⁷ of the native sequence replaced by a Ser¹⁷ residue and Asp²⁷ of the native sequence replaced by a Ser²⁷ residue.

[0014] EP 0 256 843 entitled "Expression of G-CSF and Muteins Thereof and Their Uses" reportedly discloses a modified DNA sequence encoding G-CSF wherein the N-terminus is modified for enhanced expression of protein in recombinant host cells, without changing the amino acid sequence of the protein.

[0015] EP 0 243 153 entitled "Human G-CSF Protein Expression" reportedly discloses G-CSF to be modified by inactivating at least one yeast KEX2 protease processing site for increased yield in recombinant production using yeast.

[0016] Shaw, US Patent No. 4,904,584, entitled "Site-Specific Homogeneous Modification of Polypeptides" reportedly discloses lysine altered proteins.

[0017] WO/9012874 reportedly discloses cysteine altered variants of proteins.

[0018] Australian Patent Application Document No. AU-A-10948/92, entitled, "Improved Activation of Recombinant Proteins" reportedly discloses the addition of amino acids to either terminus of a G-CSF molecule for the purpose of aiding in the folding of the molecule after prokaryotic expression.

[0019] Australian Patent Application Document No. AU-A-76380/91, entitled, "Muteins of the Granulocyte Colony Stimulating Factor (G-CSF)" reportedly discloses muteins of G-CSF in the sequence Leu-Gly-His-Ser-Leu-Gly-Ile at position 50-56 of G-CSF with 174 amino acids, and position 53 to 59 of the G-CSF with 177 amino acids, and/or at least one of the four histidine residues at positions 43, 79, 156 and 170 of the mature G-CSF with 174 amino acids or at positions 46, 82, 159, or 173 of the mature G-CSF with 177 amino acids.

[0020] GB 2 213 821, entitled "Synthetic Human Granulocyte Colony Stimulating Factor Gene" reportedly discloses a synthetic G-CSF-encoding nucleic acid sequence incorporating restriction sites to facilitate the cassette mutagenesis of selected regions, and flanking restriction sites to facilitate the incorporation of the gene into a desired expression system.

[0021] US Patent No. 5,214,132 reportedly discloses the modification of human G-CSF at amino acid positions 1, 3, 4, 5, and 17 [see, also, Kuga *et al.*, *Biochem. Biophys. Res. Commun.* 159:103-111 (1989)].

[0022] US Patent No. 5,218,092 reportedly discloses the modification of human G-CSF at amino acid positions 1, 3, 4, 5, 17, 145, and 147.

[0023] US Patent No. 5,581,476 discloses the three-dimensional structure of G-CSF to the atomic level. From this three-dimensional structure, one can forecast with substantial certainty how changes in the composition of a G-CSF molecule may result in structural changes. These structural characteristics may be correlated with biological activity to design and produce G-CSF analogs.

[0024] Signal transduction, the way in which G-CSF affects cellular metabolism, is not currently thoroughly understood. In general, G-CSF binds to and activates the G-CSF cell-surface receptor through conformational changes. This binding thereby initiates a signaling cascade (e.g., recruiting kinases to the cytoplasmic domain) which apparently initiates the changes within particular progenitor cells, leading to cellular responses such as differentiation, proliferation and migration. The G-CSF/G-CSFR complex is thought to undergo endocytic trafficking processes of internalization and sorting to recycling or degradation [Lauffenburger, and Linderman, *Receptors: Models for Binding, Trafficking, and Signaling*, New York: Oxford University Press (1993)].

[0025] Endocytic uptake of G-CSF potentiates intracellular proteolytic cytokine degradation in endosomal and/or lysosomal compartments. Internalized cytokine receptors if not recycled can be destroyed. Thus, endocytic trafficking could cause the depletion of G-CSF from the extracellular medium, as well as the downregulation of the G-CSF receptor. Accordingly, it would be beneficial to alter the G-CSF structure in a manner that retains proper receptor activation for signal transduction, but diminishes endocytic internalization and/or enhances endosomal sorting to recycling rather than degradation [Lauffenburger *et al.*, *Scratching The (Cell) Surface: Cytokine Engineering For Improved Ligand/Receptor Trafficking Dynamics*, *Chem & Biol* 5:R257-R263 (1988)].

[0026] Thus, there is a need to develop better therapeutic ligands of G-CSF. Such agents would have longer half-lives and induce greater cellular proliferation if the ligand were not as prone to endocytosis and subsequent lysosomal degradation. Accordingly, it is an object of the present invention to provide these ligands and methods for producing and testing them.

SUMMARY OF THE INVENTION

[0027] In this invention, we describe an approach to improve G-CSF potency through designed improvements in cellular trafficking. It has been found that the substitutions of the present invention result in G-CSF analogs having an effect on cellular trafficking as compared to G-CSF of SEQ ID NO: 2 or recombinant G-CSF having a methionine residue at position -1 ("metG-CSF" or "r-met-HuG-CSF"). Unless otherwise indicated herein, these species are referred to collectively as "wild-type". The effects from these modifications demonstrate advantages in stability and potency, which are not seen in other G-CSF species. In particular, these analogs provide enhanced cellular response (G-CSF agonist type activity). Such changes in cellular response occur by affecting G-CSF receptor binding and/or the processes of sorting, recycling and degradation via the ligand/receptor endocytic trafficking pathways.

[0028] The present invention relates to human G-CSF analog polypeptides which comprise an amino acid substitution in the sequence of SEQ ID NO: 2 selected from the group consisting of: 1) a substitution of aspartic acid with histidine at position number 109, [His¹⁰⁹]G-CSF; 2) a substitution of aspartic acid with histidine at position number 112, [His¹¹²]G-CSF; 3) a substitution of glutamine with histidine at position number 119; [His¹¹⁹]G-CSF; and 4) any of the said polypeptide analogs optionally including an N-terminal methionyl residue. Such analogs may additionally be derivatized with one or more water soluble polymers.

[0029] In yet another aspect, the invention provides polynucleotides encoding human G-CSF analog polypeptides described above. Presently preferred polynucleotides are set out in the DNA sequences of SEQ ID NOS: 3, 5, or 7 (and complementary strands) and include those additionally encoding an N-terminal methionyl residue.

[0030] In still another aspect, the invention comprehends an expression construct containing a polynucleotide as set

out above.

[0031] Furthermore, the invention provides a host cell containing a polynucleotide as set out above. Presently preferred host cells are selected from the group consisting of bacteria, mammalian cells, tumor cells, yeast cells, and insect cells.

[0032] In yet another aspect, the present invention provides a process for producing G-CSF analog polypeptides [His¹⁰⁹]G-CSF, [His¹¹²]G-CSF, [His¹¹⁹]G-CSF, and the Met¹ species thereof from a host cell containing nucleic acid encoding such analogs, wherein said process comprises: culturing said host cell containing a polypeptide as set out above under conditions facilitating the expression of such polypeptide; and obtaining such G-CSF analog polypeptide.

[0033] The invention also provides for pharmaceutical compositions comprising a G-CSF analog polypeptide, as set out above, and a pharmaceutically acceptable carrier. These compositions may be employed in methods for treating a hematopoietic, neurological or reproduction related conditions comprised of administering an effective amount of a composition as set out above to a patient in need thereof. Such conditions include reduced hematopoietic function, reduced immune function, reduced neutrophil count, reduced neutrophil mobilization, mobilization of peripheral blood progenitor cells, sepsis, severe chronic neutropenia, bone marrow transplants, infectious diseases, leucopenia, thrombocytopenia, anemia, enhancing engraftment of bone marrow during transplantation, enhancing bone marrow recovery in treatment of radiation, chemical or chemotherapeutic induced bone marrow aplasia or myelosuppression, and acquired immune deficiency syndrome.

[0034] In still another aspect, the invention provides a method of sensitizing cells to chemotherapy and radiotherapy comprised of administering an effective amount of a pharmaceutical composition as set out above to a patient in need thereof

[0035] The invention also provides a method for culturing hematopoietic cells *in vitro* comprising: placing said cells in a suitable culture medium, said suitable culture medium containing a G-CSF analog polypeptide as set out above; and providing suitable conditions for the growth of said hematopoietic cells.

[0036] The invention also provides a method as set out above wherein said treatment, sensitizing, or culturing includes the use of at least one additional factor selected from among EPO, G-CSF, SCF, M-GDF, GM-CSF, M-CSF, CSF-1, IL-1, IL-2, IL-3, EL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, interleukins, IGF-1, LIF, interferon, a neurotrophic factor, flt-3/flk-2 ligand, and a fibroblast growth factor. The invention correspondingly provides a kit containing components for culturing hematopoietic cells comprised of: any of the polypeptide analogs as set out above; components suitable for preparing medium for culturing hematopoietic cells; and, optionally, at least one additional factor as set out above.

DETAILED DESCRIPTION OF THE INVENTION

[0037] G-CSF has been found to be useful in the treatment of various conditions where an increase in neutrophils will provide benefits. For example, for cancer patients, G-CSF is beneficial as a means of selectively stimulating neutrophil production to compensate for hematopoietic deficits resulting from chemotherapy or radiation therapy. Other indications include treatment of various infectious diseases and related conditions, such as sepsis, which is typically caused by a metabolite of bacteria. G-CSF is also useful alone, or in combination with other compounds, such as other cytokines, for growth or expansion of cells in culture (for example, for bone marrow transplants or *ex vivo* expansion). G-CSF has been administered to transplant patients as an adjunct to treatment of infection or for treatment of neutropenia. However, it is rapidly cleared through receptor-mediated endocytosis by peripheral neutrophils and precursor cells in bone marrow expressing G-CSFR, and thus, the potency of the drug is reduced by this negative feedback mechanism. Because cells naturally express G-CSFR in low numbers, decreasing the endosomal affinity of the complex may not only reduce receptor downregulation but may also enhance ligand recycling, as predicted by modeling. Therefore, G-CSF drug potency would be improved by mutations to G-CSF that would enhance trafficking properties.

[0038] The present invention addresses novel G-CSF analog polypeptides which demonstrate advantages in stability which are not seen in other G-CSF species. In particular, these analogs provide enhanced cellular response (superagonist or G-CSF agonist type activity) as compared to wild-type G-CSF. Such changes in cellular response occur by affecting G-CSF receptor binding and/or the processes of sorting, recycling and degradation via the ligand/receptor endocytic trafficking pathways.

[0039] All analog changes of the present invention are based on the 174 amino acid sequence for G-CSF in SEQ ID NO: 2. One skilled in the art will appreciate that the analogs of the present invention may also be constructed with an N-terminal methionine residue.

[0040] The section headings are used herein for organizational purposes only, and are not to be construed as in any way limiting the subject matter described.

A. Role of G-CSF in the Treatment of Neutropenia

[0041] G-CSF has been found to be useful in the treatment of conditions where an increase in neutrophils will provide benefits. For example, for cancer patients, G-CSF is beneficial as a means of selectively stimulating neutrophil production

to compensate for hematopoietic deficits resulting from chemotherapy or radiation therapy. Other indications include treatment of various infectious diseases and related conditions, such as sepsis, which is typically caused by a metabolite of bacteria. G-CSF is also useful alone, or in combination with other compounds, such as other cytokines, for growth or expansion of cells in culture (for example, for bone marrow transplants or *ex vivo* expansion). G-CSF has been administered to transplant patients as an adjunct to treatment of infection or for treatment of neutropenia [Diflo *et al.*, *Hepatology* 16:PA278 (1992); Wright *et al.*, *Hepatology* 14:PA48 (1991); Lachaux *et al.*, *J. Ped.* 123:1005-1008 (1993); Colquehoun *et al.*, *Transplantation* 56:755-758 (1993)].

[0042] The term "neutropenia" refers to a condition with an abnormally low number of neutrophils in the peripheral circulation. The neutrophil arises from the bone marrow and is fully mature when it is released into the circulation. It is then fully prepared to function in its role as the first line of cellular defense. It becomes activated, i.e., able to perform many of its functions, by exposing it to certain pro-inflammatory mediators and the chemotactic factors that attract it to the site of an inciting stimulus. Severe neutropenia can be life-threatening, because it can lead to severe infections.

[0043] Alternately, the term "neutrophilia" refers to a condition with an abnormally high number of neutrophils in the peripheral circulation. Neutrophilia, or an increased white blood count, has many causes, including exposure to extreme cold, heat, recent surgery, physical trauma, infection of any type, burns, shocks, tumors, non-infection inflammation, such as gout, arthritis, thyroid problems, and drugs.

B. G-CSF Analogs of the Present Invention

[0044] The present invention contemplates the production of analogs of wild-type G-CSF polypeptides and their encoding polypeptides wherein: 1) histidine is substituted for aspartic acid at position 109, [His¹⁰⁹]G-CSF; 2) histidine is substituted for aspartic acid at position 112, [His¹¹²]G-CSF; and 3) histidine is substituted for glutamine at position 119, [His¹¹⁹]G-CSF, and the Met⁻¹ species thereof. The above substitutions are according to the numbering of SEQ ID NO: 2. The amino acid sequences for these three analogs can be found in SEQ ID NOS: 4, 6, and 8, respectively. The present invention contemplates the production of human G-CSF analog polypeptides that have an increased potency relative to wild-type G-CSF to stimulate cellular proliferation, and increased half-lives. Such changes in cellular response occur by affecting G-CSF receptor binding and/or the processes of sorting, recycling, and degradation via the ligand/receptor endocytic trafficking pathways.

[0045] These analogs were developed by means of a general, computational framework for the design of ligands, using single substitutions to histidine, which act as pH-activated switches to improve their cellular trafficking properties. The strategy described herein exploits the pH difference between the extracellular medium and endosomal compartments as a switch for histidine protonation to reduce the endosomal affinity of the ligand-receptor complex, and this is predicted to enhance recycling of intact ligand back into the extracellular medium.

[0046] The selection of residues for mutation to histidine was guided by electrostatic considerations. The intention was to create G-CSF analogs that would bind G-CSFR with close to (or, if possible, greater) wild-type affinity for neutral histidine but that would bind poorly for positively charged histidine. At acidic pH, the binding affinity would be decreased from that at neutral pH by the cost of deprotonating histidine in unbound G-CSF [Yang and Honig, *J. Mol. Biol.* 231: 459-474 (1993)]. If the pK_a of histidine in the unbound protein was 6.5 [Tanokura, *Biochim. Biophys. Acta.* 742:576-585 (1982)], this would result in 10-fold lower affinity at pH 5.5.

[0047] Candidate histidine mutations were identified by a three-part procedure *in silico*. First, the nature of electrostatic binding interactions was quantified using a method [Kangas and Tidor, *J. Chem. Phys.* 109:7522-7545 (1998)] that explicitly accounts for ligand desolvation and interaction and locates regions at the interface that are somewhat too negative or too positive for optimal binding. Second, trial histidine G-CSF analogs (both neutral and positively charged) were constructed and energy minimized. Third, the electrostatic binding free energy of each was computed.

[0048] The concept detailed herein involves novel mutants of G-CSF which were rationally chosen. The crystal structure of the complex between G-CSF and its receptor, G-CSFR, was utilized to calculate the net electrostatic potential at the major binding interface between ligand and receptor. Amino acid residues on G-CSF which contributed residual net electrostatic potential were chosen as candidates for mutagenesis. The predominance of overly negative regions indicates excessive negative charge density, including locations corresponding to the charged residues Glu¹⁹, Asp¹⁰⁹, and Asp¹¹² as well as the polar residues Gln²⁰, Thr¹¹⁶, and Gln¹¹⁹, indicating that there is insufficient corresponding positive charge density from the receptor. Electrostatic contributions to binding may be enhanced with decreased negative charge on the ligand in these six sites; neutral histidine may be tolerated in the complex, but positively charged histidine could repel the positive charge density of the receptor. To test these sites, individual histidine mutants were constructed computationally on the identified residues.

[0049] Using the three dimensional crystallographic structure of the complex between G-CSF and its receptor (G-CSFR) the net electrostatic potential at the major binding interface between ligand and receptor can be calculated. See US Patent No. 5,581,476 (incorporated by reference herein); Aritomi *et al.*, *Nature* 401(6754):713-718 (1999). Amino acid residues on G-CSF which contributed residual net electrostatic potential were chosen as candidates for mutagenesis.

Six such residues on G-CSF were identified: Asp¹¹², Asp¹⁰⁹, Gln¹¹⁹, Gln²⁰, Thr¹¹⁶, and Glu¹⁹. In particular, three of the proposed single histidine substitutions have been made and tested: Asp¹⁰⁹His ([His¹⁰⁹]G-CSF), Asp¹¹²His ([His¹¹²]G-CSF), and Gln¹¹⁹His ([His¹¹⁹]G-CSF) (using the numbering of SEQ ID NO: 2 with the methionine at -1).

[0050] The rationale for the histidine mutagenesis of G-CSF was to affect the cellular trafficking of G-CSF and/or G-CSFR. Upon binding to G-CSFR on the surface of a cell, G-CSF is internalized into the cell and a sorting decision is made in endosomal vesicles. The components of the complex can either be degraded in the lysosomes or recycled back to the surface intact. It is known in many other ligand/receptor systems that when the ligand and receptor remain in complex, they are preferentially degraded; however, if the complex dissociates in the endosomes, there is enhanced recycling of the ligand and/or receptor. At the cell surface, the pH of the environment is roughly 7.0; in the endosomes, the pH is around 5.0 - 6.0. Since the amino acid histidine is the only residue which is expected to titrate in this pH range, the histidine substitution is expected to affect cellular trafficking properties. More specifically, single histidine mutants alter trafficking properties based upon the differences in electrostatic binding free energy at pH 7.0 versus pH 5.0, due solely to protonation of the mutated histidine at pH 5.0.

[0051] Specifically contemplated by the present invention is site-specific mutagenesis of genomic, cDNA, and synthetic DNA sequences of wild-type G-CSF polypeptide. Presently preferred polynucleotide sequences of the invention include the DNA sequences of SEQ ID NO: 3 [His¹⁰⁹]G-CSF; SEQ ID NO: 5 [His¹¹²]G-CSF; SEQ ID NO: 7 [His¹¹⁹]G-CSF; and the Met⁻¹ species thereof. These DNA sequences may also be modified to encode another version of G-CSF having at least one of the hematopoietic biological properties of naturally occurring human G-CSF. Preferably the biological property is the property of binding to a G-CSF receptor, but another biological property is the capability of stimulating the proliferation of hematopoietic cells. Other biological properties will be apparent to those skilled in the art (See, also, Souza, *supra*).

[0052] These DNA sequences may incorporate codons facilitating transcription and translation of mRNA in microbial hosts. Such manufacturing sequences may readily be constructed according to the methods well-known in the art. See, also, Alton *et al.*, PCT published application WO 83/04053. The DNAs above may also optionally encode an N-terminal methionyl residue.

[0053] DNA sequences provided by the invention are useful in generating new and useful viral and plasmid DNA vectors, new and useful transformed and transfected procaryotic and eucaryotic host cells (including bacterial, yeast, and mammalian cells grown in culture), and new and useful methods for cultured growth of such host cells capable of expression of the present G-CSF analogs. The DNA sequences encoding biologically active G-CSF analogs herein (or corresponding RNAs) may be useful for gene therapy in instances where underproduction of G-CSF would be alleviated, or the need for increased levels of G-CSF.

[0054] The present invention also provides for processes for recombinant DNA production of the present G-CSF analogs. Provided is a process for producing the G-CSF analogs from a host cell containing nucleic acid encoding such analogs comprised of: a) culturing said host cell containing nucleic acid encoding such G-CSF analogs under conditions facilitating the expression of such DNA molecule; and b) obtaining such G-CSF analogs.

[0055] One may optionally purify and isolate such G-CSF analogs from other components obtained in the process. Methods for purifying can be found in US Patent No. 5,849,883. Other methods are well-known in the art (See, also, Souza, *supra*).

[0056] Host cells may be prokaryotic or eukaryotic and include bacteria, mammalian cells (such as Chinese Hamster Ovary (CHO) cells, monkey cells, baby hamster kidney cells, cancer cells or other cells), yeast cells, and insect cells. Preferred for greatest ease in commercial production is production using a bacterial host cell.

C. Protein Production

[0057] Given the above disclosure of human G-CSF analog polypeptides, it will be possible for one of skill in the art to produce human G-CSF analog polypeptides by automated peptide synthesis, by recombinant techniques or both.

[0058] The human G-CSF analogs of the invention can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Stewart and Young, Solid Phase Peptide Synthesis, 2d. ed., Pierce Chemical Co. (1984); Tam *et al.*, *J. Am. Chem. Soc.* 105:6442 (1983); Merrifield, *Science* 232: 341-347 (1986); and Barany and Merrifield, The Peptides, Gross and Meienhofer (eds.), Academic Press, New York, 1-284 (1979). The active protein can be readily synthesized and then screened in screening assays designed to identify reactive peptides.

[0059] Alternatively, a variety of expression vector/host systems may be utilized to contain and express a human G-CSF analog coding sequence. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (*e.g.*, baculovirus); plant cell systems transfected with virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with bacterial expression vectors (*e.g.*, Ti or pBR322 plasmid); or animal cell systems. Mammalian cells that are useful in recombinant

protein productions include but are not limited to VERO cells, HeLa cells, Chinese hamster ovary (CHO) cell lines, COS cells (such as COS-7), W138, BHK, HepG2, 3T3, RIN, MDCK, A549, PC12, K562 and 293 cells. Exemplary protocols for the recombinant expression of the protein are described herein below.

[0060] A yeast system may be employed to generate the human G-CSF analogs of the present invention. The coding region of the human G-CSF analog cDNA is amplified by PCR. A DNA encoding the yeast pre-pro-alpha leader sequence is amplified from yeast genomic DNA in a PCR reaction using one primer containing nucleotides 1-20 of the alpha mating factor gene and another primer complementary to nucleotides 255-235 of this gene [Kurjan and Herskowitz, *Cell* 30: 933-943 (1982)]. The pre-pro-alpha leader coding sequence and human G-CSF analog coding sequence fragments are ligated into a plasmid containing the yeast alcohol dehydrogenase (ADH2) promoter, such that the promoter directs expression of a fusion protein consisting of the pre-pro-alpha factor fused to the mature human G-CSF analog polypeptide. As taught by Rose and Broach [*Meth. Enz.* 185:234-279, Goeddel (ed.), Academic Press, Inc., San Diego, CA (1990)], the vector further includes an ADH2 transcription terminator downstream of the cloning site, the yeast "2-micron" replication origin, the yeast leu-2d gene, the yeast REP and REP2 genes, the *E. coli* beta-lactamase gene, and an *E. coli* origin of replication. The beta-lactamase and leu-2d genes provide for selection in bacteria and yeast, respectively. The leu-2d gene also facilitates increased copy number of the plasmid in yeast to induce higher levels of expression. The REP 1 and REP2 genes encode proteins involved in regulation of the plasmid copy number.

[0061] The DNA construct described in the preceding paragraph is transformed into yeast cells using a known method, e.g., lithium acetate treatment [Stearns *et al.*, *Meth. Enz.* 185: 280-297 (1990)]. The ADH2 promoter is induced upon exhaustion of glucose in the growth media [Price *et al.*, *Gene* 55:287 (1957)]. The pre-pro-alpha sequence effects secretion of the fusion protein from the cells. Concomitantly, the yeast KEX2 protein cleaves the pre-pro sequence from the mature human G-CSF analogs [Bitter *et al.*, *Proc. Natl. Acad. Sci. USA* 81:5330-5334 (1954)].

[0062] Alternatively, human G-CSF analogs may be recombinantly expressed in yeast using a commercially available expression system, e.g., the Pichia Expression System (Invitrogen, San Diego, CA), following the manufacturer's instructions. This system also relies on the pre-pro-alpha sequence to direct secretion, but transcription of the insert is driven by the alcohol oxidase (AOX1) promoter upon induction by methanol.

[0063] The secreted human G-CSF analog is purified from the yeast growth medium by, e.g., the methods used to purify human G-CSF analog from bacterial and mammalian cell supernatants.

[0064] Alternatively, the cDNA encoding human G-CSF analogs may be cloned into the baculovirus expression vector pVL1393 (PharMingen, San Diego, CA). This human G-CSF analog-containing vector is then used according to the manufacturer's directions (PharMingen) to infect *Spodoptera frugiperda* cells in sF9 protein-free media and to produce recombinant protein. The protein is purified and concentrated from the media using a heparin-Sepharose column (Pharmacia, Piscataway, NJ) and sequential molecular sizing columns (Amicon, Beverly, MA), and resuspended in PBS. SDS-PAGE analysis shows a single band and confirms the size of the protein, and Edman sequencing on a Proton 2090 Peptide Sequencer confirms its N-terminal sequence.

[0065] Alternatively, the human G-CSF analogs may be expressed in an insect system. Insect systems for protein expression are well-known to those of skill in the art. In one such system, *Autographa californica nuclear polyhedrosis virus* (AcNPV) is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in *Trichoplusia* larvae. The human G-CSF analog coding sequence is cloned into a nonessential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of human G-CSF analog will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein coat. The recombinant viruses are then used to infect *S. frugiperda* cells or *Trichoplusia* larvae in which human G-CSF analog is expressed [Smith *et al.*, *J. Virol.* 46: 584 (1983); Engelhard *et al.*, *Proc. Natl. Acad. Sci. USA* 91: 3224-7 (1994)].

[0066] In another example, the DNA sequence encoding the mature form of the protein is amplified by PCR and cloned into an appropriate vector, for example, pGEX-3X (Pharmacia, Piscataway, NJ). The pGEX vector is designed to produce a fusion protein comprising glutathione-S-transferase (GST), encoded by the vector, and a protein encoded by a DNA fragment inserted into the vector's cloning site. The primers for the PCR may be generated to include, for example, an appropriate cleavage site.

[0067] The recombinant fusion protein may then be cleaved from the GST portion of the fusion protein. The pGEX-3X/human G-CSF analog construct is transformed into *E. coli* XL-1 Blue cells (Stratagene, La Jolla CA), and individual transformants are isolated and grown. Plasmid DNA from individual transformants is purified and partially sequenced using an automated sequencer to confirm the presence of the desired human G-CSF analog encoding gene insert in the proper orientation.

[0068] While certain embodiments of the present invention contemplate producing the human G-CSF analog protein using synthetic peptide synthesizers and subsequent FPLC analysis and appropriate refolding of the cysteine double bonds, it is contemplated that recombinant protein production also may be used to produce the human G-CSF analog peptide compositions. For example, induction of the GST/human G-CSF analog fusion protein is achieved by growing the transformed XL-1 Blue culture at 37°C in LB medium (supplemented with carbenicillin) to an optical density at wavelength 600 nm of 0.4, followed by further incubation for 4 hours in the presence of 0.5 mM Isopropyl β-D-Thio-

lactopyranoside (Sigma Chemical Co., St. Louis MO).

[0069] The fusion protein, expected to be produced as an insoluble inclusion body in the bacteria, may be purified as follows. Cells are harvested by centrifugation; washed in 0.15 M NaCl, 10 mM Tris, pH 8, 1 mM EDTA; and treated with 0.1 mg/mL lysozyme (Sigma Chemical Co.) for 15 min. at room temperature. The lysate is cleared by sonication, and cell debris is pelleted by centrifugation for 10 min. at 12,000 X g. The fusion protein-containing pellet is resuspended in 50 mM Tris, pH 8, and 10 mM EDTA, layered over 50% glycerol, and centrifuged for 30 min. at 6000 X g. The pellet is resuspended in standard phosphate buffered saline solution (PBS) free of Mg⁺⁺ and Ca⁺⁺. The fusion protein is further purified by fractionating the resuspended pellet in a denaturing SDS polyacrylamide gel (Sambrook *et al.*, *supra*). The gel is soaked in 0.4 M KCl to visualize the protein, which is excised and electroeluted in gel-running buffer lacking SDS. If the GST/human G-CSF analog fusion protein is produced in bacteria as a soluble protein, it may be purified using the GST Purification Module (Pharmacia Biotech).

[0070] The fusion protein may be subjected to digestion to cleave the GST from the mature human G-CSF analog protein. The digestion reaction (20-40 µg fusion protein, 20-30 units human thrombin [4000 U/mg (Sigma) in 0.5 mL PBS] is incubated 16-48 hrs. at room temperature and loaded on a denaturing SDS-PAGE gel to fractionate the reaction products. The gel is soaked in 0.4 M KCl to visualize the protein bands. The identity of the protein band corresponding to the expected molecular weight of the human G-CSF analog may be confirmed by partial amino acid sequence analysis using an automated sequencer (Applied Biosystems Model 473A, Foster City, CA).

[0071] Alternatively, the DNA sequence encoding the predicted mature human G-CSF analog protein may be cloned into a plasmid containing a desired promoter and, optionally, a leader sequence [see, e.g., Better *et al.*, *Science* 240: 1041-43 (1988)]. The sequence of this construct may be confirmed by automated sequencing. The plasmid is then transformed into *E. coli*, strain MC1061, using standard procedures employing CaCl₂ incubation and heat shock treatment of the bacteria (Sambrook *et al.*, *supra*). The transformed bacteria are grown in LB medium supplemented with carbenicillin, and production of the expressed protein is induced by growth in a suitable medium. If present, the leader sequence will affect secretion of the mature human G-CSF analog protein and be cleaved during secretion.

[0072] The secreted recombinant protein is purified from the bacterial culture media by the method described herein below.

[0073] Mammalian host systems for the expression of the recombinant protein also are well-known to those of skill in the art. Host cell strains may be chosen for a particular ability to process the expressed protein or produce certain post-translation modifications that will be useful in providing protein activity. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation and acylation. Post-translational processing, which cleaves a "prepro" form of the protein, may also be important for correct insertion, folding and/or function. Different host cells, such as CHO, HeLa, MDCK, 293, WI38, and the like, have specific cellular machinery and characteristic mechanisms for such post-translational activities, and may be chosen to ensure the correct modification and processing of the introduced foreign protein.

[0074] In a particularly preferred method of recombinant expression of the human G-CSF analog proteins of the present invention, 293 cells are co-transfected with plasmids containing the human G-CSF analog cDNA in the pCMV vector (5' CMV promoter, 3'HGH poly A sequence) and pSV2neo (containing the neo resistance gene) by the calcium phosphate method. Preferably, the vectors should be linearized with Scal prior to transfection. Similarly, an alternative construct using a similar pCMV vector with the neo gene incorporated can be used. Stable cell lines are selected from single cell clones by limiting dilution in growth media containing 0.5 mg/mL G418 (neomycin-like antibiotic) for 10-14 days. Cell lines are screened for human G-CSF analog expression by ELISA or Western blot, and high-expressing cell lines are expanded for large scale growth.

[0075] It is preferable that the transformed cells are used for long-term, high-yield protein production and as such stable expression is desirable. Once such cells are transformed with vectors that contain selectable markers along with the desired expression cassette, the cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The selectable marker is designed to confer resistance to selection, and its presence allows growth and recovery of cells that successfully express the introduced sequences. Resistant clumps of stably transformed cells can be proliferated using tissue culture techniques appropriate to the cell.

[0076] A number of selection systems may be used to recover the cells that have been transformed for recombinant protein production. Such selection systems include, but are not limited to, HSV thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase genes, in tk-, hgp^{rt}- or ap^{rt}- cells, respectively. Also, anti-metabolite resistance can be used as the basis of selection for dhfr, that confers resistance to methotrexate; gpt, that confers resistance to mycophenolic acid; neo, that confers resistance to the aminoglycoside, G418; also, that confers resistance to chlorsulfuron; and hyg^r, that confers resistance to hygromycin. Additional selectable genes that may be useful include trpB, which allows cells to utilize indole in place of tryptophan, or hisD, which allows cells to utilize histinol in place of histidine. Markers that give a visual indication for identification of transformants include anthocyanins, β-glucuronidase and its substrate, GUS, and luciferase and its substrate, luciferin.

D. Protein Purification

[0077] It will be desirable to purify the human G-CSF analog proteins generated by the present invention. Protein purification techniques are well-known to those of skill in the art. These techniques involve, at one level, the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. Having separated the polypeptide from other proteins, the polypeptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, exclusion chromatography, polyacrylamide gel electrophoresis, and isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or even HPLC.

[0078] Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of an encoded protein or peptide. The term "purified protein or peptide" as used herein, is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state. A purified protein or peptide therefore also refers to a protein or peptide, free from the environment in which it may naturally occur.

[0079] Generally, "purified" will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95% or more of the proteins in the composition.

[0080] Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptide within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a "-fold purification number." The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification, and whether or not the expressed protein or peptide exhibits a detectable activity.

[0081] Various techniques suitable for use in protein purification will be well-known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies, and the like; heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

[0082] There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed, utilizing an HPLC apparatus, will generally result in a greater "-fold" purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

[0083] It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE [Capaldi *et al.*, *Biochem. Biophys. Res. Comm.* 76:425 (1977)]. It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

[0084] One may optionally purify and isolate such G-CSF analogs from other components obtained in the process. Methods for purifying can be found in US Patent No. 5,849,883. Other methods are well-known in the art (See, also, Souza, *supra*, each incorporated herein by reference). These documents describe specific exemplary methods for the isolation and purification of G-CSF compositions that may be useful in isolating and purifying the G-CSF analogs of the present invention. Given the disclosure of these patents, it is evident that one of skill in the art would be well aware of numerous purification techniques that may be used to purify G-CSF from a given source.

[0085] Also it is contemplated that a combination of anion exchange and immunoaffinity chromatography may be employed to produce purified G-CSF analog compositions of the present invention.

E. Vectors for Cloning, Gene Transfer and Expression

[0086] As discussed in the previous section, expression vectors are employed to express the human G-CSF analog polypeptide product, which can then be purified and used for the treatment of neutropenia. In other embodiments, expression vectors may be used in gene therapy applications to introduce the human G-CSF analog-encoding nucleic

acids into cells in need thereof and/or to induce human G-CSF analog expression in such cells. The present section is directed to a description of the production of such expression vectors.

[0087] Expression requires that appropriate signals be provided in the vectors, which include various regulatory elements, such as enhancers/promoters from both viral and mammalian sources that drive expression of the genes of interest in host cells. Elements designed to optimize messenger RNA stability and translatability in host cells also are described. The conditions for the use of a number of dominant drug selection markers for establishing permanent, stable cell clones expressing the products also are provided, as is an element that links expression of the drug selection markers to expression of the polypeptide.

a. Regulatory Elements

[0088] **Promoters and Enhancers.** Throughout this application, the term "expression construct" or "expression vector" is meant to include any type of genetic construct containing a nucleic acid coding for gene products in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. In certain embodiments, expression includes both transcription of a gene and translation of mRNA into a gene product. The nucleic acid encoding a gene product is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

[0089] The term "promoter" will be used herein to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized is derived from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

[0090] At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

[0091] Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

[0092] The particular promoter employed to control the expression of a nucleic acid sequence of interest is not believed to be important, so long as it is capable of directing the expression of the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

[0093] In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, β -actin, rat insulin promoter, the phosphoglycerol kinase promoter, and glyceraldehyde-3-phosphate dehydrogenase promoter, all of which are promoters well-known and readily available to those of skill in the art, can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or mammalian cellular or bacterial phage promoters that are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose. By employing a promoter with well-known properties, the level and pattern of expression of the protein of interest following transfection or transformation can be optimized.

[0094] Selection of a promoter that is regulated in response to specific physiologic or synthetic signals can permit inducible expression of the gene product. Several inducible promoter systems are available for production of viral vectors. One such system is the ecdysone system (Invitrogen, Carlsbad, CA), which is designed to allow regulated expression of a gene of interest in mammalian cells. It consists of a tightly-regulated expression mechanism that allows virtually no basal level expression of the transgene, but over 200-fold inducibility.

[0095] Another inducible system that would be useful is the Tet-Off™ or Tet-On™ system (Clontech, Palo Alto, CA), originally developed by Gossen and Bujard [Gossen and Bujard, *Proc. Natl. Acad. Sci. USA* 15;89(12):5547-51 (1992); Gossen *et al.*, *Science* 268(5218):1766-9 (1995)].

[0096] In some circumstances, it may be desirable to regulate expression of a transgene in a gene therapy vector. For example, different viral promoters with varying strengths of activity may be utilized depending on the level of expression desired. In mammalian cells, the CMV immediate early promoter is often used to provide strong transcriptional activation. Modified versions of the CMV promoter that are less potent have also been used when reduced levels of expression of the transgene are desired. When expression of a transgene in hematopoietic cells is desired, retroviral promoters such as the LTRs from MLV or MMTV are often used. Other viral promoters that may be used depending on the desired effect include SV40, RSV LTR, HIV-1 and HIV-2 LTR, adenovirus promoters, such as from the E1A, E2A, or MLP region, AAV LTR, cauliflower mosaic virus, HSV-TK, and avian sarcoma virus.

[0097] Similarly, tissue specific promoters may be used to effect transcription in specific tissues or cells, so as to reduce potential toxicity or undesirable effects to non-targeted tissues. For example, promoters such as the PSA, probasin, prostatic acid phosphatase, or prostate-specific glandular kallikrein (hK2) may be used to target gene expression in the prostate.

[0098] In certain indications, it may be desirable to activate transcription at specific times after administration of the gene therapy vector. This may be done with such promoters as those that are hormone or cytokine regulatable. For example, in gene therapy applications where the indication is a gonadal tissue where specific steroids are produced or routed to, use of androgen- or estrogen-regulated promoters may be advantageous. Such promoters that are hormone-regulatable include MMTV, MT-1, ecdysone, and RuBisco. Other hormone-regulated promoters, such as those responsive to thyroid, pituitary, and adrenal hormones, are expected to be useful in the present invention. Cytokine and inflammatory protein responsive promoters that could be used include K and T Kininogen [Kageyama *et al.*, *J. Biol. Chem.* 262(5):2345-51 (1987)], c-fos, TNF-alpha, C-reactive protein [Arcone *et al.*, *Nucleic Acids Res.* 16(8):3195-207 (1988)], haptoglobin [Oliviero *et al.*, *EATBO J.* 6(7):1905-12 (1987)], serum amyloid A2, C/EBP alpha, IL-1, IL-6 [Poli and Cortese, *Proc. Natl. Acad. Sci. USA* 86(21):8202-6 (1989)], complement C3 [Wilson *et al.*, *Mol. Cell. Biol.* 10(12):6181-91 (1990)], IL-8, alpha-1 acid glycoprotein [Prowse and Baumann, *Mol. Cell. Biol.* 8(1):42-51 (1988)], alpha-1 antitrypsin, lipoprotein lipase [Zechner *et al.*, *Mol. Cell. Biol.* 8(6):2394-401 (1988)], angiotensinogen [Ron *et al.*, *Mol. Cell. Biol.* 11(5):2887-95 (1991)], fibrinogen, c-jun (inducible by phorbol esters, TNF-alpha, UV radiation, retinoic acid, and hydrogen peroxide), collagenase (induced by phorbol esters and retinoic acid), metallothionein (heavy metal and glucocorticoid inducible), stromelysin (inducible by phorbol ester, interleukin-1 and EGF), alpha-2 macroglobulin and alpha-1 antichymotrypsin.

[0099] Other promoters that could be used according to the present invention include Lac-regulatable, heat (hyperthermia) inducible promoters, and radiation-inducible, *e.g.*, EGR [Joki *et al.*, *Hum. Gene Ther.* 6(12):1507-13 (1995)], alpha-inhibin, RNA pol III tRNA met, and other amino acid promoters, U1 snRNA [Bartlett *et al.*, *Proc. Natl. Acad. Sci. USA* 20;93(17):8852-7 (1996)], MC-1, PGK, beta-actin and alpha-globin. Many other promoters that may be useful are listed in Walther and Stein [*J. Mol. Med.* 74(7):379-92 (1996)].

[0100] It is envisioned that any of the above promoters, alone or in combination with another, may be useful according to the present invention depending on the action desired. In addition, this list of promoters should not be construed to be exhaustive or limiting, and those of skill in the art will know of other promoters that may be used in conjunction with the promoters and methods disclosed herein.

[0101] Another regulatory element contemplated for use in the present invention is an enhancer. These are genetic elements that increase transcription from a promoter located at a distant position on the same molecule of DNA. Enhancers are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins. The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization. Enhancers useful in the present invention are well-known to those of skill in the art and will depend on the particular expression system being employed [Scharf *et al.*, *Results Probl. Cell. Differ.* 20: 125-62 (1994); Bittner *et al.*, *Meth. Enzymol.* 153: 516-544 (1987)].

[0102] **Polyadenylation Signals.** Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human or bovine growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read-through from the cassette into other sequences.

[0103] **IRES.** In certain embodiments of the invention, the use of internal ribosome entry site (IRES) elements is contemplated to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5' methylated Cap dependent translation and begin translation at internal sites [Pelletier and Sonenberg, *Nature* 334:320-325 (1988)]. IRES elements from two members of the picornavirus family (poliovirus and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988, *supra*), as well as an IRES from a mammalian message [Macejak

and Sarnow, *Nature* 353:90-94 (1991)]. IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

[0104] Any heterologous open reading frame can be linked to IRES elements. This includes genes for secreted proteins, multi-subunit proteins, encoded by independent genes, intracellular or membrane-bound proteins, and selectable markers. In this way, the expression of several proteins can be simultaneously engineered into a cell with a single construct and a single selectable marker.

b. Delivery of Expression Vectors

[0105] There are a number of ways in which expression vectors may be introduced into cells. In certain embodiments of the invention, the expression construct comprises a virus or engineered construct derived from a viral genome. In other embodiments, non-viral delivery is contemplated. The ability of certain viruses to enter cells via receptor-mediated endocytosis, to integrate into host cell genome, and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells [Ridgeway, In: Vectors: A survey of molecular cloning vectors and their uses, Rodríguez & Denhardt (eds.), Stoneham: Butterworth, 467-492, 1988; Nicolas and Rubenstein, In: Vectors: A survey of molecular cloning vectors and their uses, Rodríguez & Denhardt (eds.), Stoneham: Butterworth, 493-513, 1988; Baichwal and Sugden, In: Gene Transfer, Kuchelapati (ed.), New York, Plenum Press, 117-148, 1986; Temin, In: Gene Transfer, Kuchelapati (ed.), New York: Plenum Press, 149-188, 1986)]. The first viruses used as gene vectors were DNA viruses including the papovaviruses (simian virus 40, bovine papilloma virus, and polyoma) (Ridgeway, 1988, *supra*; Baichwal and Sugden, 1986, *supra*) and adenoviruses (Ridgeway, 1988, *supra*; Baichwal and Sugden, 1986, *supra*). These have a relatively low capacity for foreign DNA sequences and have a restricted host spectrum. Furthermore, their oncogenic potential and cytopathic effects in permissive cells raise safety concerns. They can accommodate only up to 8 kb of foreign genetic material but can be readily introduced in a variety of cell lines and laboratory animals (Nicolas and Rubenstein, 1988, *supra*; Temin, 1986, *supra*).

[0106] It is now widely recognized that DNA may be introduced into a cell using a variety of viral vectors. In such embodiments, expression constructs comprising viral vectors containing the genes of interest may be adenoviral (see, for example, US Patent No. 5,824,544; US Patent No. 5,707,618; US Patent No. 5,693,509; US Patent No. 5,670,488; US Patent No. 5,585,362), retroviral (see for example, US Patent No. 5,888,502; US Patent No. 5,830,725; US Patent No. 5,770,414; US Patent No. 5,686,278; and US Patent No. 4,861,719), adeno-associated viral (see for example, US Patent No. 5,474,935; US Patent No. 5,139,941; US Patent No. 5,622,856; US Patent No. 5,658,776; US Patent No. 5,773,289; US Patent No. 5,789,390; US Patent No. 5,834,441; US Patent No. 5,863,541; US Patent No. 5,851,521; and US Patent No. 5,252,479), an adenoviral-adenoassociated viral hybrid (see, for example, US Patent No. 5,856,152) or a vaccinia viral or a herpes viral (see, for example, US Patent No. 5,879,934; US Patent No. 5,849,571; US Patent No. 5,830,727; US Patent No. 5,661,033; and US Patent No. 5,328,688) vector.

[0107] There are a number of alternatives to viral transfer of genetic constructs. This section provides a discussion of methods and compositions of non-viral gene transfer. DNA constructs of the present invention are generally delivered to a cell, and in certain situations, the nucleic acid or the protein to be transferred may be transferred using non-viral methods.

[0108] Several non-viral methods for the transfer of expression constructs into cultured mammalian cells are contemplated by the present invention. These include calcium phosphate precipitation [Graham and Van Der Eb, *Virology* 52: 456-467 (1973); Chen and Okayama, *Mol. Cell. Biol.* 7:2745-2752 (1987); Rippe *et al.*, *Mol. Cell. Biol.* 10:689-695 (1990)], DEAE-dextran [Gopal, *Mol. Cell. Biol.*, 5:1188-1190 (1985)], electroporation [Tur-Kaspa *et al.*, *Mol. Cell. Biol.* 6:716-718 (1986); Potter *et al.*, *Proc. Natl. Acad. Sci. USA* 81:7161-7165 (1984)], direct microinjection [Harland and Weintraub, *J. Cell Biol.* 101:1094-1099 (1985)], DNA-loaded liposomes [Nicolau and Sene, *Biochim. Biophys. Acta.* 721:185-190 (1982); Fraley *et al.*, *Proc. Natl. Acad. Sci. USA* 76:3348-3352 (1979); Felgner, *Sci. Am.* 276(6):102-106 (1997); Felgner, *Hum. Gene Ther.* 7(15):1791-3 (1996)], cell sonication [Fechheimer *et al.*, *Proc. Natl. Acad. Sci. USA* 84:8463-8467 (1987)], gene bombardment using high velocity microprojectiles [Yang *et al.*, *Proc. Natl. Acad. Sci. USA* 87:9568-9572 (1990)], and receptor-mediated transfection [Wu and Wu, *J. Biol. Chem.* 262:4429-4432 (1987); Wu and Wu, *Biochem.*, 27:887-892 (1988); Wu and Wu, *Adv. Drug Deliv. Rev.* 12:159-167 (1993)].

[0109] Once the construct has been delivered into the cell, the nucleic acid encoding the therapeutic gene may be positioned and expressed at different sites. In certain embodiments, the nucleic acid encoding the therapeutic gene may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation via homologous recombination (gene replacement), or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of, or in synchronization with, the host cell cycle. How the expression construct is delivered to

a cell, and where in the cell the nucleic acid remains, is dependent on the type of expression construct employed.

[0110] In a particular embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers [Ghosh and Bachhawat, In: Liver diseases, targeted diagnosis and therapy using specific receptors and ligands, Wu & Wu (ed.), New York: Marcel Dekker, pp. 87-104 (1991)]. The addition of DNA to cationic liposomes causes a topological transition from liposomes to optically birefringent liquid-crystalline condensed globules [Radler *et al.*, *Science* 275(5301):810-4 (1997)]. These DNA-lipid complexes are potential non-viral vectors for use in gene therapy and delivery.

[0111] Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful. Also contemplated in the present invention are various commercial approaches involving "lipofection" technology. In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA [Kaneda *et al.*, *Science* 243:375-378 (1989)]. In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) [Kato *et al.*, *J Biol. Chem.* 266:3361-3364 (1991)]. In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression constructs have been successfully employed in transfer and expression of nucleic acid *in vitro* and *in vivo*. Then they are applicable for the present invention.

[0112] Other vector delivery systems that can be employed to deliver a nucleic acid encoding a therapeutic gene into cells include receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993, *supra*).

[0113] Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1957, *supra*) and transferrin [Wagner *et al.*, *Proc. Natl. Acad. Sci. USA* 87(9):3410-3414 (1990)]. Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle [Ferkol *et al.*, *FASEB J.* 7:1081-1091 (1993); Perales *et al.*, *Proc. Natl. Acad. Sci. USA* 91:4086-4090 (1994)] and epidermal growth factor (EGF) has also been used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

[0114] In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau *et al.* [Methods *Enzymol.* 149:157-176 (1987)] employed lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. Thus, it is feasible that a nucleic acid encoding a therapeutic gene also may be specifically delivered into a particular cell type by any number of receptor-ligand systems with or without liposomes.

[0115] In another embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above that physically or chemically permeabilize the cell membrane. This is applicable, particularly for transfer *in vitro*; however, it may be applied for *in vivo* use as well. Dubensky *et al.* [Proc. Nat. Acad. Sci. USA 81:7529-7533 (1984)] successfully injected polyomavirus DNA in the form of CaPO₄ precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif [Proc. Natl. Acad. Sci. USA 83:9551-9555 (1986)] also demonstrated that direct intraperitoneal injection of CaPO₄-precipitated plasmids results in expression of the transfected genes.

[0116] Another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them [Klein *et al.*, *Nature* 327:70-73 (1987)]. Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force [Yang *et al.*, Proc. Natl. Acad. Sci. USA 87: 9568-9572 (1990)]. The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

F. Methods of Treating Neutropenia

[0117] As mentioned herein above, it is contemplated that the human G-CSF analogs or the vectors comprising polynucleotides encoding such proteins will be employed in replacement therapy protocols for the treatment of neutropenia. G-CSF has been found to be useful in the treatment of various conditions, where an increase in neutrophils will provide benefits. For example, for cancer patients, G-CSF is beneficial as a means of selectively stimulating neutrophil production to compensate for hematopoietic deficits resulting from chemotherapy or radiation therapy. Other indications

include treatment of various infectious diseases and related conditions, such as sepsis, which is typically caused by a metabolite of bacteria. G-CSF is also useful alone, or in combination with other compounds, such as other cytokines, for growth or expansion of cells in culture (for example, for bone marrow transplants or *ex vivo* expansion). G-CSF has been administered to transplant patients as an adjunct to treatment of infection or for treatment of neutropenia.

a. Protein-Based Therapy

[0118] One of the therapeutic embodiments of the present invention is the provision, to a subject in need thereof, compositions comprising the human G-CSF analogs of the present invention. As discussed above, the proteins may have been generated through recombinant means or by automated peptide synthesis. The human G-CSF analog formulations for such a therapy may be selected based on the route of administration and may include liposomal formulations as well as classic pharmaceutical preparations.

[0119] The human G-CSF analog proteins are formulated into appropriate preparation and administered to one or more sites within the subject in a therapeutically-effective amount. In particularly preferred embodiments, the human G-CSF analog protein-based therapy is effected continuous or intermittent intravenous administration. By "therapeutically-effective amount" the present invention refers to that amount of a human G-CSF analog that is sufficient to support an observable change in the level of one or more biological activities of G-CSF. The change may be either an increase level of G-CSF activity. Preferably, the change is an increase in neutrophil proliferation.

[0120] Those of skill in the art will understand that the amounts of human G-CSF analog for therapeutic use may vary. It is contemplated that the specific activity of the human G-CSF analog protein preparation may be in the range of about 100 units/mg of protein to about 500 units/mg protein. Thus, a given preparation of a human G-CSF analog may comprise about 100 units/mg protein, about 125 units/mg protein, about 150 units/mg protein, about 175 units/mg protein, about 200 units/mg protein, about 225 units/mg protein, about 250 units/mg protein, about 275 units/mg protein, about 300 units/mg protein, about 325 units/mg protein, about 350 units/mg protein, about 375 units/mg protein, about 400 units/mg protein, about 425 units/mg protein, about 450 units/mg protein, about 475 units/mg protein and about 500 units/mg protein. A particularly preferred range is from about 100 units/mg protein to about 200 units/mg protein; a more preferable range is between about 150 to about 200 units/mg protein. Preferably, the protein composition is substantially free of contaminating factors, contamination level of less than 0.02% (w/w). Human G-CSF analog compositions, suitable for injection into a patient, can be prepared, for example, by reconstitution with a pharmacologically acceptable diluent of a lyophilized sample comprising purified human G-CSF analog and stabilizing salts.

[0121] Administration of the compositions can be systemic or local, and may comprise a single site injection of a therapeutically-effective amount of the human G-CSF analog protein composition. Any route known to those of skill in the art for the administration of a therapeutic composition of the invention is contemplated including, for example, intravenous, intramuscular, subcutaneous or a catheter for long-term administration. Alternatively, it is contemplated that the therapeutic composition may be delivered to the patient at multiple sites. The multiple administrations may be rendered simultaneously or may be administered over a period of several hours. In certain cases, it may be beneficial to provide a continuous flow of the therapeutic composition. Additional therapy may be administered on a period basis, for example, daily, weekly, or monthly.

b. Combination Therapy

[0122] In addition to therapies based solely on the delivery of the human G-CSF analogs, combination therapy is specifically contemplated. In the context of the present invention, it is contemplated that the human G-CSF analog therapy could be used similarly in conjunction with other agents commonly used for the treatment of neutropenia.

[0123] To achieve the appropriate therapeutic outcome, using the methods and compositions of the present invention, one would generally provide a composition comprising the human G-CSF analog and at least one other therapeutic agent (second therapeutic agent). In the present invention, it is contemplated that the second therapeutic agent may involve the administration or inclusion of at least one additional factor selected from among EPO, G-CSF, M-GDF, SCF, GM-CSF, M-CSF, CSF-1, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, or other various interleukins, IGF-1, LIF, interferon (such as α , β , gamma or consensus), neurotrophic factors (such as BDNF, NT-3, CTNF or noggin), other multi-potent growth factors (such as, to the extent these are demonstrated to be such multi-potent growth factors, flt-3/flk-2 ligand, stem cell proliferation factor, and totipotent stem cell factor), fibroblast growth factors (such as FGF), and analogs, fusion molecules, or other derivatives of the above. For example, G-CSF in combination with SCF has been found to mobilize peripheral blood progenitor cells *in vivo*. *Ex vivo*, for example, G-CSF in combination with SCF, IL-3 and IL-6 has been found useful for expansion of peripheral blood cells. Likewise, the present G-CSF analogs will provide for similar uses.

[0124] The combination therapy compositions would be provided in a combined amount effective to produce the desired therapeutic outcome in the treatment of neutropenia. This process may involve contacting the cells with the

human G-CSF analog and the second agent(s) or factor(s) at the same time. This may be achieved by administering a single composition or pharmacological formulation that includes both agents, or by administering two distinct compositions or formulations, at the same time, wherein one composition includes the human G-CSF analog therapeutic composition and the other includes the second therapeutic agent.

[0125] Alternatively, the human G-CSF analog treatment may precede or follow the other agent treatment by intervals ranging from minutes to weeks. In embodiments where the second therapeutic agent and the human G-CSF analog are administered separately, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the second agent and the human G-CSF analog would still be able to exert an advantageously combined effect. In such instances, it is contemplated that one would administer both modalities within about 12-24 hours of each other and, more preferably, within about 6-12 hours of each other, with a delay time of only about 12 hours being most preferred. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations. [0126] Systemic delivery of human G-CSF analog expression constructs or proteins to patients may be a very efficient method for delivering a therapeutically effective gene to counteract the immediate clinical manifestations of the disease. Alternatively, local delivery of the human G-CSF analog and/or the second therapeutic agent may be appropriate in certain circumstances.

G. Assays for Determining G-CSF Analog Activity and Binding

[0127] In certain aspects of the present invention, it may be necessary to determine the activity of human G-CSF analog. In particular, the effect of the therapeutic compositions of the present invention on neutropenia may need to be monitored. Those of skill in the art are aware of numerous assays to determine G-CSF activity, some of which are described in the present section. This is by no means intended to be an exhaustive list of such assays and is merely intended to provide certain exemplary assays well-known to those of skill in the art that may be used in determining the G-CSF activity of the present invention. Further, the present section also describes assays for the determination of G-CSF binding to its receptor. Exemplary *in vitro* and *in vivo* assays for determining these activities are provided herein below.

a. *In Vitro* Assays

[0128] **Cellular Proliferation Assays.** Cellular proliferation assays, some of which are described herein, are used to determine G-CSF analog effect on inducing cellular proliferation. Many assays are well-known in the art and some of these assays are described as follows.

[0129] **Cell Counting.** Cells are passaged into supplemented MEM α medium (without G-CSF) 24 h prior to the initiation of the cell proliferation experiment, at which point parallel flasks of cells at a density of 10^5 cells/mL are incubated in MEM α medium with 125 pM wild-type G-CSF or G-CSF analogs. Cell growth in each flask is measured on days 2, 5, and 8 as follows. Briefly, cells are diluted in an isotonic solution (ISOTON II, Coulter Diagnostics, Hialeah, FL), and counted in a Coulter counter (Coulter Electronics, Hialeah, FL).

[0130] **^3H -Thymidine Incorporation.** This assay is based upon the incorporation of labeled ^3H -thymidine into newly synthesized DNA of cells upon cell division. Some researchers also use the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) instead of [^3H]-thymidine in proliferation assays. Briefly, cells are passaged into supplemented MEM α medium (without G-CSF) 24 hr prior to the initiation of the cellular proliferation experiment, at which point parallel wells/flasks of cells at a density of 10^5 cells/mL are incubated in MEM α medium with 125 pM wild-type G-CSF or G-CSF analogs. ^3H -thymidine is added to the cell culture 12-24 hr prior to counting (termination of the experiment), and at the end of the assay the amount of radioactive incorporation in the DNA is measured in a liquid scintillation counter. Details of this method are well-known to one skilled in the art.

[0131] **MTT.** MTT is used for the quantitative determination of cellular proliferation and activation e.g. in response to growth factors and cytokines. It is also used for the quantification of antiproliferative or cytotoxic effects e.g. mediated by tumor necrosis factor- α or - β and for the measurement of interferon action. The assay is based on the cleavage of the yellow tetrazolium salt, MTT, to purple formazan crystals by metabolic active cells. These salt crystals are insoluble in aqueous solution, but may be solubilized by adding the solubilization solution included in the kit and incubating the plates overnight in humidified atmosphere (e.g. 37°C, 6.5% CO $_2$). The solubilized formazan product is spectrophotometrically quantified using an ELISA reader. An increase in number of living cells results in an increase in the total metabolic activity in the sample. This increase directly correlates to the amount of purple formazan crystals formed, as monitored by the absorbance. Commercial MTT assay can be purchased from Roche Diagnostics (Indianapolis, IN).

[0132] **Ligand Depletion Assay.** Ligand depletion for each protein is measured over time as described herein. As in the above experiments, G-CSF-dependent (OCI/AML1) cells are passaged into supplemented MEM α medium (without G-CSF) 24 h prior to the initiation of the ligand depletion experiments, at which point parallel flasks of cells at a density

of 10^5 cells/mL are incubated in MEM α medium with 125 pM wild-type G-CSF or G-CSF analog. After 24 h, the cell number in each flask is measured, as described above, and an aliquot of each medium supernatant, obtained after centrifugation to pellet cellular debris, is stored at -20°C for measurement of G-CSF concentration. This is repeated every 24 h for eight days. The concentrations of G-CSF in the medium supernatant samples are then quantitated using

enzyme-linked immunosorbent assay (ELISA) kits obtained from R&D Systems (Minneapolis, MN).

[0133] Internalization and Recycling Experiments. Internalization experiments are performed over a time period of 5 min., similarly to published work [Kuwabara *et al.*, *Amer. J. Physiol. Endocrinol. Metabol.* 32: E1-E9 (1995)]. Briefly, 10^8 cells are washed twice with PBS and then incubated on ice in labeled ligand for 30 min. to obtain surface complexes. The cells are again washed twice with ice-cold PBS and resuspended in MEM α , at 37°C at $t=0$. The change in surface complexes and internal complexes is followed for a time period of 5 min.; a plot of internal complexes versus the time integral of surface complexes yields a linear relationship, the slope of which is the complex internalization rate constant [Wiley *et al.*, *J. Biol. Chem.* 257: 4222-4229 (1982)]. Recycling experiments are performed identically to internalization experiments, except over a time period of 25 min.. Data from recycling experiments are parameter fitted to obtain recycling rate constants.

[0134] Binding Assays. Molecular binding assays, some of which are described herein, are used to assess G-CSF analog binding to G-CSFR. These *in vitro* and cell-based assays are described as follows.

[0135] BIAcore. Ligand binding affinity of G-CSF analog to G-CSF receptor is measured using a BIAcore®2000 (BIAcore, Inc., Piscataway, NJ). Histidine-tagged wild-type G-CSF is immobilized on the chip surface, and free receptor (- 0.25 - 10 nM) is passed over the chip to generate a standard equilibrium curve and to calculate wild-type binding affinity using a 1:1 binding model. To determine each mutant binding affinity, 2 nM free receptor is mixed with a known concentration of mutant ligand and passed over the chip. Mutant equilibrium binding affinities are determined, using a 1:1 binding model with competition. Binding affinity data is analyzed using BIA evaluation 3.1 software (BIAcore, Inc.); and equilibrium dissociation constants (K_D) are determined.

[0136] ELISA. Binding of G-CSF mutant analogs to G-CSFR is measured in an ELISA format competition assay. In this assay, G-CSFR is captured with the non-neutralizing G-CSF receptor antibody LMM741 (PharMingen, San Diego, CA) per manufacturer's protocol. Analog proteins are then assayed for their ability to compete with HRP-labeled G-CSF for receptor binding.

b. *In Vivo* Assays

[0137] Before the human G-CSF analog compositions of the present invention are employed in human therapeutic protocols, it may be desirable to monitor the effects of such compositions in animal models. There are a number of animal models that may be used in *in vivo* assays, known to those of skill in the art, that may be useful in the present invention.

[0138] In order to determine the efficacy of the human G-CSF analog protein and gene therapy compositions of the present invention, such animals may be injected intramuscularly and/or intravenously with the compositions of the present invention, and the ability to stimulate neutrophil proliferation in the presence and absence of the compositions may be determined. Such determinations will be helpful in providing guidance on the dosages and times of administration and the efficacy of a given composition against neutropenia. In gene therapy protocols, immunofluorescent staining of sections, obtained from biopsied muscle, may be performed, and expression of the human G-CSF analog in the transduced muscle fibers may be determined.

H. Pharmaceutical Compositions

[0139] The present invention also comprehends pharmaceutical compositions comprising effective amounts of polypeptide products of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers useful in G-CSF therapy. Such compositions include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., thimersol, benzyl alcohol), and bulking substances (e.g., lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds, such as polylactic acid, polyglycolic acid, etc., or in association with liposomes. Such compositions will influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the present G-CSF analogs. See, e.g., *Remington's Pharmaceutical Sciences*, 18th Ed. (1990) Mack Publishing Co., Easton, PA, pages 1435-1712.

[0140] Derivatives of the present G-CSF analogs are also comprehended herein. Such derivatives include molecules modified by one or more water soluble polymer molecules, such as polyethylene glycol, or by the addition of polyamino acids, including fusion proteins (procedures for which are well-known in the art). Such derivatization may occur singularly at the N- or C-terminus or there may be multiple sites of derivatization. Substitution of one or more amino acids with

lysine may provide additional sites for derivatization. (See US Patent No.: 5,824,784 and US Patent No.: 5,824,778.

[0141] The present analogs or derivatives thereof may be formulated for injection, or oral, nasal, pulmonary, topical, or other types of administration as one skilled in the art will recognize. The formulation may be liquid or may be solid, such as lyophilized, for reconstitution.

[0142] In general, the present analogs (or derivatives thereof) will be useful in the same way that currently available G-CSFs are useful, except the present analogs provide enhanced cellular response (superagonist or G-CSF agonist activity). Such changes in cellular response occur by affecting G-CSF receptor binding and/or the processes of sorting, recycling and degradation via the ligand/receptor endocytic trafficking pathways.

[0143] These uses include the treatment of a variety of hematopoietic, neurological, and reproduction related conditions. Thus, the present compositions and methods for the manufacture of medicaments may be useful for the treatment of such conditions. Conditions alleviated or modulated by the administration of the present analogs are typically those characterized by a reduced hematopoietic or immune function and more specifically, a reduced neutrophil count. Such conditions may be induced as a course of therapy for other purposes, such as chemotherapy or radiation therapy. Such conditions may result from infectious disease, such as bacterial, viral, fungal, or other infectious disease. For example, sepsis results from bacterial infection. Or, such condition may be hereditary or environmentally caused, such as severe chronic neutropenia or leukemias. Age may also play a factor, as in the geriatric setting; patients may have a reduced neutrophil count or reduced neutrophil mobilization. Some of such conditions are reviewed in Filgrastim (r-metHuG-CSF) in: Clinical Practice, Morstyn and Dexter (eds.), Marcel Dekker, Inc., New York (1993), p. 351. Other less-studied conditions which may be alleviated or modulated by the administration of the present analogs may include the reduction of lipids (or cholesterol) in the blood stream and certain cardiovascular conditions, as G-CSF may induce the production of plasminogen activators. In addition, the present G-CSF analog compositions may be used for mobilization of peripheral blood progenitor cells.

[0144] The present G-CSF analogs (or derivative compositions) may also be used *in vitro*. For example, in a gene therapy setting, one may desire to transfect a hematopoietic cell with exogenous DNA, and culture said cell using the present G-CSF analog formulations. Thus, in yet another aspect, the present invention involves a method for culturing hematopoietic cells *in vitro* comprised of: a) placing said cells in a suitable culture medium, said suitable culture medium containing a G-CSF analog composition according to the present invention, and b) providing suitable conditions for the growth of said hematopoietic cells.

[0145] More particularly, the present invention provides a method of transfecting hematopoietic cells with exogenous DNA comprising: a) culturing said hematopoietic cells with a G-CSF analog composition according to the present invention, and b) transfecting said cultured cells with exogenous DNA. The hematopoietic cells may be, for example, bone marrow cells or peripheral blood progenitor cells. In addition, other cells well-known to one skilled in the art can be used.

[0146] In order to prepare human G-CSF analog containing compositions for clinical use, it will be necessary to prepare the viral expression vectors, proteins, and nucleic acids as pharmaceutical compositions, *i.e.*, in a form appropriate for *in vivo* applications. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

[0147] One will generally desire to employ appropriate salts and buffers to render delivery vectors stable and allow for uptake by target cells. Buffers also will be employed when recombinant cells are introduced into a patient. Aqueous compositions of the present invention comprise an effective amount of the human G-CSF analog or an expression vector to cells, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well-known in the art. Except insofar as any conventional media or agent is incompatible with the vectors or cells of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

[0148] The active compositions of the present invention include classic pharmaceutical preparations. Administration of these compositions according to the present invention will be via any common route so long as the target tissue is available via that route. The pharmaceutical compositions may be introduced into the subject by any conventional method, *e.g.*, by intravenous, intradermal, intramuscular, intramammary, intraperitoneal, intrathecal, retrobulbar, intrapulmonary (*e.g.*, term release); by oral, sublingual, nasal, anal, vaginal, or transdermal delivery, or by surgical implantation at a particular site. The treatment may consist of a single dose or a plurality of doses over a period of time.

[0149] The active compounds may be prepared for administration as solutions of free base or pharmacologically acceptable salts in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

[0150] The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile

powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing; for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents (for example, sugars or sodium chloride). Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption (for example, aluminum monostearate and gelatin).

[0151] Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle that contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques that yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0152] As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well-known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

[0153] For oral administration the polypeptides of the present invention may be incorporated with excipients and used in the form of non-ingestible mouthwashes and dentifrices. A mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active ingredient may be incorporated into an antiseptic wash containing sodium borate, glycerin and potassium bicarbonate. The active ingredient may also be dispersed in dentifrices, including: gels, pastes, powders and slurries. The active ingredient may be added in a therapeutically effective amount to a paste dentifrice that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants.

[0154] The compositions of the present invention may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups also can be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

[0155] The compositions of the present invention may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups also can be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

[0156] Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration.

[0157] Generally, an effective amount of the present G-CSF analogs (or derivatives) will be determined by the age, weight, and condition or severity of disease of the recipient. See, *Remington's Pharmaceutical Sciences*, *supra*, pages 697-773, herein incorporated by reference. Typically, a dosage of between about 0.001 $\mu\text{g/kg}$ body weight/day to about 1000 $\mu\text{g/kg}$ body weight/day, may be used, but more or less, as a skilled practitioner will recognize, may be used. Dosing may be one or more times daily, or less frequently, and may be in conjunction with other compositions as described herein. It should be noted that the present invention is not limited to the dosages recited herein.

[0158] "Unit dose" is defined as a discrete amount of a therapeutic composition dispersed in a suitable carrier. For example, where polypeptides are being administered parenterally, the polypeptide compositions are generally injected in doses ranging from 1 $\mu\text{g/kg}$ to 100mg/kg body weight/day, preferably at doses ranging from 0.1mg/kg to about 50 mg/kg body weight/day. Parenteral administration may be carried out with an initial bolus followed by continuous infusion

to maintain therapeutic circulating levels of drug product. Those of ordinary skill in the art will readily optimize effective dosages and administration regimens as determined by good medical practice and the clinical condition of the individual patient.

[0159] The frequency of dosing will depend on the pharmacokinetic parameters of the agents and the routes of administration. The optimal pharmaceutical formulation will be determined by one of skill in the art depending on the route of administration and the desired dosage. See, for example, *Remington's Pharmaceutical Sciences*, supra, pages 1435-1712. Such formulations may influence the physical state, stability, rate of *in vivo* release and rate of *in vivo* clearance of the administered agents. Depending on the route of administration, a suitable dose may be calculated according to body weight, body surface areas or organ size. Further refinement of the calculations necessary to determine the appropriate treatment dose is routinely made by those of ordinary skill in the art without undue experimentation, especially in light of the dosage information and assays disclosed herein, as well as the pharmacokinetic data observed in animals or human clinical trials.

[0160] Appropriate dosages may be ascertained through the use of established assays for determining level of neutropenia in conjunction with relevant dose-response data. The final dosage regimen will be determined by the attending physician, considering factors that modify the action of drugs, e.g., the drug's specific activity, severity of the damage and the responsiveness of the patient, the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. As studies are conducted, further information will emerge regarding appropriate dosage levels and duration of treatment for specific diseases and conditions.

[0161] In gene therapy embodiments employing viral delivery, the unit dose may be calculated in terms of the dose of viral particles being administered. Viral doses include a particular number of virus particles or plaque forming units (pfu). For embodiments involving adenovirus, particular unit doses include 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , 10^8 , 10^9 , 10^{10} , 10^{11} , 10^{12} , 10^{13} or 10^{14} pfu. Particle doses may be somewhat higher (10 to 100-fold) due to the presence of infection-defective particles.

[0162] It will be appreciated that the pharmaceutical compositions and treatment methods of the invention may be useful in fields of human medicine and veterinary medicine. Thus the subject to be treated may be a mammal, preferably human or other animal. For veterinary purposes, subjects include for example, farm animals including cows, sheep, pigs, horses and goats, companion animals such as dogs and cats, exotic and/or zoo animals, laboratory animals including mice, rats, rabbits, guinea pigs and hamsters; and poultry such as chickens, turkeys, ducks and geese.

[0163] In addition, the present invention contemplates a kit containing components for culturing bone marrow cells or peripheral blood progenitor cells comprised of: a) a G-CSF analog composition of the present invention; and b) components suitable for preparing medium for culturing bone marrow cells or peripheral blood progenitor cells.

I. Examples

[0164] The present invention is described in more detail with reference to the following non-limiting examples, which are offered to more fully illustrate the invention, but are not to be construed as limiting the scope thereof. The examples illustrate the preparation of the present G-CSF analogs and the testing of these analogs *in vitro*. Those of skill in the art will understand that the techniques described in these examples represent techniques described by the inventors to function well in the practice of the invention, and as such constitute preferred modes for the practice thereof. However, it should be appreciated that those of skill in the art should in light of the present disclosure, appreciate that many changes can be made in the specific methods that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Design Rationale for Histidine Mutants

[0165] G-CSF mutants were designed based on the principle that histidine titration could maintain relatively tight binding on the cell surface but lead to weaker binding in endosomal compartments. Taking advantage of the pH decrease from approximately 7 at the cell surface to between 5 and 6 in endosomes, mutants were designed to largely maintain (or improve) the electrostatic interactions at extracellular pH but worsen the interaction at endosomal pH. The pK_a of histidine on the surface of free ligand (~ 6.5) [Tanokura, *Biochim. Biophys. Acta*. 742:576-585 (1982)] suggests that, given the appropriate local protein environment, histidine titration could result in large pH-dependent effects on binding between extracellular and endosomal media. Calculations were used to identify candidate locations for histidine substitution. Preferred locations were those for which the computed binding affinity was comparable to wild-type for neutral histidine and significantly weaker for positively charged histidine.

EXAMPLE 2

Preparation and Characterization of GCSF Analogs

[0166] G-CSF analogs were prepared by either insertional or site-directed mutagenesis of DNA encoding r-met-HuG-CSF using the PCR overlap extension method [Aiyar *et al.*, *Meth. Mol. Biol.* 57:177-191 (1996)]. After confirming mutations by sequence analysis, each of the mutants was expressed in *E. coli* K12, refolded, and purified as described in [Lu *et al.*, *J. Biol. Chem.* 267:8770-8777 (1992)]. For protocols and procedures [see, also, "Recombinant PCR," Russell Higuchi, In: *PCR Protocols*, Innis, Gelfand, Sninsky, and White (eds.), Academic Press, Inc., San Diego, CA (1990); and "Site-directed mutagenesis of cloned DNA", In: *Molecular Cloning, A Lab Manual*, Sambrook, Fritsch, and Maniatis (eds.), Cold Spring Harbor Press, CSH, N.Y. (1989)]. The *E. coli* expression of r-met-HuG-CSF was previously reported. See US Patent No. 4,810,643 and US Patent No. 5,549,883. The DNA encoding recombinant human G-CSF had an initial methionine codon followed by codons for the 174 amino acid species of human G-CSF. The purified r-met-HuG-CSF analogs retain the initiating Met (position Met -1).

[0167] The amino acid sequence and nucleic acid sequence for [His¹⁰⁹]G-CSF are shown in SEQ ID NOS: 3 (DNA) and 4 (amino acid). The amino acid sequence and nucleic acid sequence for [His¹¹²]G-CSF are shown in SEQ ID NOS: 5 (DNA) and 6 (amino acid). The amino acid sequence and nucleic acid sequence for [His¹¹⁹]G-CSF are shown in SEQ ID NOS: 7 (DNA) and 8 (amino acid).

[0168] Confirmation of the identity of the G-CSF analogs of the present invention was accomplished by N-terminal amino acid sequencing of intact proteins. Sequences of the purified G-CSF analogs matched the sequences predicted from the respective DNA sequences such as those shown in SEQ ID NOS: 3, 5, and 7. Such methods are well-known in the art [Shively, *EXS* 88:99-117 (2000)].

Structure Preparation of Histidine Mutants

[0169] The X-ray crystal structure of G-CSF in 2:2 complex with the ligand-binding domain of G-CSFR was solved by Arntoni *et al.* [*Nature* 401:713-717 (1999)] using data to 2.8 Å from crystals grown and maintained at pH 7.5 [Arntoni *et al.*, *Acta Crystallogr. D Biol. Crystallogr.* 56:751-753 (2000)]. The structure (entry 1CD9) was obtained from the Protein Data Bank (www.rcsb.org/pdb/) [Berman *et al.*, *Nucl. Acids Res.* 28:235-242 (2000)]. The calculations described herein focus on the interface formed by segments A and B, which is the major binding interface involving one G-CSF molecule (A) and one CRH domain of G-CSFR (B). The coordinates for these chains were extracted from the coordinate file and polar and aromatic hydrogen atom positions were built using CHARMM [Brooks *et al.*, *J. Comput. Chem.* 4:187-217 (1983); Brunger and Karplus, *Proteins* 4:148-156 (1988)]. Additionally, a small number of missing heavy atom positions, all distant from the interface, were built in standard geometry using CHARMM. Examination of titratable side chains at the interface in the wild-type complex, none of which were histidine, suggested leaving all in their standard neutral pH titration states.

In Silico Mutagenesis of G-CSF for Histidine Mutants

[0170] The potential sites for histidine mutations were identified by a procedure *in silico*. Conformations of all ligand and receptor side chains at the binding interface within 10 Å of the mutation, including the mutation itself, were repacked onto a rigid backbone using a standard rotamer library [Dunbrack and Karplus, *J. Mol. Biol.* 230:543-574 (1993)]. The energy function incorporated van der Waals interactions and electrostatic interactions with a distance-dependent dielectric constant ($\epsilon = 4r$, where r is in Å) and no cutoff, as implemented in CHARMM19 [Brooks *et al.*, *J. Comput. Chem.* 4:187-217 (1983); Gilson and Honig, *Nature* 330:84-86 (1987)], extended to include PARSE partial atomic charges [Sitkoff *et al.*, *J. Phys. Chem.* 98:1978-1988 (1994)]. Energy-minimized complexes were found using dead-end elimination (and A*) [Desmet *et al.*, *Nature* 356:539-542 (1992); Goldstein, *Biophys. J.* 66:1335-1340 (1994)]. This procedure, when applied to the wild-type complex, correctly rebuilt the crystal structure to within the resolution of the rotamer library.

Electrostatic Calculations for Histidine Mutants

[0171] The selection of residues in G-CSF for mutations to histidine was determined by electrostatic considerations. Computational details were similar to published work [Hendsch and Tidor, *Protein Sci.* 8:1381-1392 (1999)]. Continuum electrostatic calculations were carried out by solving the linearized Poisson-Boltzmann equation with finite-difference methods using a locally modified version of the DELPHI computer program [Gilson and Honig, *Nature* 330:84-86 (1987); Gilson *et al.*, *J. Comput. Chem.* 9:327-335 (1988); Sharp and Honig, *Annu. Rev. Biophys. Biophys. Chem.* 19:301-332 (1990)]. The PARSE parameters were used for atomic radii and partial atomic charges [Sitkoff *et al.*, *J. Phys. Chem.* 98:1978-1988 (1994)]. A value of 4 was used for the protein dielectric constant and 80 for the solvent. The molecular

surface was described using a 1.4 Å probe sphere, and the bulk ionic strength was chosen to be 145 mM with a Stern layer of 2 Å. Calculations were performed using a two-step focusing procedure with 23% and 92% fill. For visualization of electrostatic potentials, a cubic lattice of 65 x 65 x 65 grid units was used. For electrostatic binding free energy calculations, a 191 x 191 x 191 grid was used (final grid spacing 0.476 Å) and each value given was the average of 10 offsets of the molecule relative to the grid. Electrostatic complementarity suggested six candidate mutation sites. The predominance of overly negative regions indicates excessive negative charge density, including locations corresponding to the charged residues Glu¹⁹, Asp¹⁰⁹, and Asp¹¹² as well as the polar residues Gln²⁰, Thr¹¹⁶, and Gln¹¹⁹.

Histidine Mutant Structure Generation

[0172] Mutant complexes, in which each of the six ligand residues (Glu¹⁹, Gln²⁰, Asp¹⁰⁹, Asp¹¹², Thr¹¹⁶, and Gln¹¹⁹) were singly mutated to histidine, were generated computationally. Each position was substituted with two neutral histidine tautomers (protonated at either the δ or ε nitrogen, indicated as His_δ⁰ or His_ε⁰, respectively) and positively charged histidine (His⁺). A constrained minimization procedure was utilized in which the mutant side chain and those of neighboring residues were given full freedom to repack using algorithms based on dead-end elimination and A* [Desmet *et al.*, *Nature* 356:539-542 (1992); Goldstein, *Biophys. J.* 66:1335-1340 (1994); Leach and Lemon, *Proteins* 33:227-239 (1998)]. The complementarity of mutant complexes was visualized and compared to wild-type.

Candidate Selection for Histidine Mutants

[0173] Candidates were selected for experimental tests based on the principle that mutants should bind receptor roughly as well as (or perhaps even better than) wild-type for the better-binding His⁰ tautomer (corresponding to cell surface conditions) and significantly more poorly than wild-type for His⁺ (corresponding to endosomal conditions). Once the difference between binding His⁰ and His⁺ was more than a few kcal/mol, there was no advantage to making it larger because it would be greater than the cost of deprotonating His⁺ in the unbound state and binding as His⁰. Thus, Asp¹⁰⁹His and Asp¹¹²His were made and purified for experimental characterization. Additionally, because Gln¹¹⁹His was predicted to bind better than wild-type on the cell surface, it was also made and purified even though it was not clear from the calculations whether the binding would be significantly weaker in endosomes.

Electrostatic Binding Free Energy Values for Wild-Type and Histidine Mutants

[0174] The electrostatic contribution to the binding free energy for wild-type and each of the His_δ⁰, His_ε⁰, and His⁺ mutants was computed using a simple rigid binding model and continuum electrostatics. While not treating all details of binding explicitly, this model is straightforward to apply and should be capable of distinguishing moderate to large binding differences among the complexes. The results show a few different behaviors. Most of the mutants show His⁰ (at least one of the histidine tautomers) binding almost but not quite as well as wild-type (up to 2.5 kcal/mol worse). Two exceptions are Glu¹⁹His⁰, which is not tolerated in the complex (computed to bind roughly 10 kcal/mol worse than wild-type) and Gln¹¹⁹His⁰, which is computed to bind somewhat better than wild-type (by 1.7 kcal/mol for the His_δ⁰ tautomer). Interestingly, Glu¹⁹ appears essential to receptor binding [Reidhaar-Olson *et al.*, *Biochemistry* 35:9034-9041 (1996); Layton *et al.*, *J. Biol. Chem.* 274:17445-17451 (1999)]. In a cell proliferation assay, Glu¹⁹Ala elicits essentially no response (data not shown), consistent with this calculation. For three of the mutants [Glu¹⁹His ([His¹⁹]G-CSF), Asp¹⁰⁹His ([His¹⁰⁹]G-CSF), and Asp¹¹²His ([His¹¹²]G-CSF)], the pH-dependence of binding is predicted to change in the desired direction because His⁺ has a computed binding free energy roughly 5 or more kcal/mol worse than His⁰ (and more than 7 kcal/mol worse than wild-type). For the other three mutants [Gln²¹His ([His²¹]G-CSF), Thr¹¹⁷His ([His¹¹⁷]G-CSF), and Gln¹¹⁹His ([His¹¹⁹]G-CSF)] the difference in binding between His⁰ and His⁺ is too close to be confident about the predictions (less than 1 kcal/mol).

EXAMPLE 3

Experimental Materials and Cell Culture

[0175] Minimum essential medium alpha (MEMα), L-glutamine, penicillin-streptomycin, and fetal bovine serum (FBS) were obtained from Life Technologies, Inc. (Rockville, MD). Isotonic solution for the Coulter counter (ISOTON II, Coulter Diagnostics, Hialeah, FL) was obtained from Curtin Matheson Scientific Inc. (Houston, TX).

[0176] The G-CSF-dependent suspension cell line, OCI/AML1, a generous gift from Ernest A. McCulloch [Ontario Cancer Institute (OCI), Princess Margaret Hospital, 610 University Avenue, Toronto, Ontario, M5G 2M9, Canada], was used for all experiments. Cells were routinely cultured in Corning 75-cm² tissue culture flasks in MEMα supplemented with 20% FBS, 200 mM L-glutamine, 100 units/mL penicillin, 100 g/mL streptomycin, and 270 pM G-CSF in a humidified

atmosphere with 5% CO₂. Cells were passaged to 10⁵ cells/mL every 3 to 4 days.

EXAMPLE 4

5 Experimental Results with Histidine Mutants

Cell Proliferation Assay

10 [0177] All three histidine mutants - [His¹⁰⁹]G-CSF, [His¹¹²]G-CSF, and [His¹¹⁹]G-CSF - were compared to wild-type G-CSF in a G-CSF-dependent cell proliferation assay. OCI/AML1 cells were passaged into supplemented MEM α medium (without G-CSF) 24 h prior to the initiation of the cell proliferation experiment, at which point parallel flasks of cells at a density of 10⁵ cells/mL were incubated in MEM α medium with 125 pM wild-type G-CSF, [His¹⁰⁹]G-CSF, [His¹¹²]G-CSF, or [His¹¹⁹]G-CSF. Cell growth in each flask was measured on days 2, 5, and 8 using a Coulter counter. After two days, no differences in cell proliferation could be detected. However, after five days, [His¹¹⁹]G-CSF significantly increased
15 cellular proliferation. By day eight, the cells treated with [His¹¹⁹]G-CSF were almost double the number of control, and cells treated with [His¹⁰⁹]G-CSF and [His¹¹²]G-CSF showed smaller, but significant increases over control. Therefore, all mutants were more potent than wild-type G-CSF in promoting cell proliferation, despite the fact that all of the mutated residues lie directly at the interface with the receptor.

20 *Ligand Depletion*

[0178] Ligand depletion for each protein was measured over time. As in the above experiments, OCI/AML1 cells were passaged into supplemented MEM α medium (without G-CSF) 24 h prior to the initiation of the ligand depletion experiments, at which point parallel flasks of cells at a density of 10⁵ cells/mL were incubated in MEM α medium with 125 pM
25 wild-type G-CSF, [His¹⁰⁹]G-CSF, [His¹¹²]G-CSF, or [His¹¹⁹]G-CSF. After 24 h, the cell number in each flask was measured as described above and an aliquot of each medium supernatant, obtained after centrifugation to pellet cellular debris, was stored at -20C for measurement of G-CSF concentration. This was repeated every 24 h for eight days. The concentrations of G-CSF, [His¹⁰⁹]G-CSF, [His¹¹²]G-CSF, and [His¹¹⁹]G-CSF in the medium supernatant samples were quantitated using enzyme-linked immunosorbent assay (ELISA) kits obtained from R&D Systems (Minneapolis, MN).
30 Each supernatant sample was assayed in duplicate. Both Asp \rightarrow His mutants, which were predicted to be the best in terms of trafficking properties, resulted in half-lives at least 10 times that of wild-type G-CSF. Indeed, it appears that the earlier-noted enhancement in cell proliferation of both Asp \rightarrow His mutants ([His¹⁰⁹]G-CSF and [His¹¹²]G-CSF) by day 8 relative to wild-type is a direct result of having enough ligand available to stimulate the cells. Additionally, the Gln \rightarrow His mutant, [His¹¹⁹]G-CSF, had a half-life greater than 6-fold that of wild-type. This was significant since the potency of this
35 mutant resulted in almost twice as many cells as wild-type by day 8, and therefore one might expect cellular trafficking of this mutant to be greater than wild-type.

[0179] At least two independent experiments were performed for both the cell proliferation and ligand depletion studies.

40 *Ligand Binding*

[0180] The ligand binding affinity of G-CSF analog to G-CSF receptor is measured using a BIAcore[®]2000 (BIAcore, Inc., Piscataway, NJ). Histidine-tagged wild-type GCSF is immobilized on the chip surface, and free receptor (- 0.25 - 10 nM) is passed over the chip to generate a standard equilibrium curve and to calculate wild-type binding affinity using
45 a 1:1 model. To determine each mutant ligand binding affinity, 2 nM free receptor is mixed with a known concentration of mutant ligand and passed over the chip. Mutant equilibrium binding affinities are determined, using a 1:1 model with competition. Binding affinity data is analyzed using BIA evaluation 3.1 software (BIAcore, Inc.); and equilibrium dissociation constants (K_D) are determined.

[0181] To determine whether the increase in half-life and potency of the mutants was attributable to higher ligand recycling rates, rate constants indicative of complex internalization, and ligand recycling were measured and reported
50 as follows.

Internalization of G-CSF Analogs

[0182] Internalization experiments were performed on two of the G-CSF analogs, [His¹⁰⁹]G-CSF, [His¹¹²]G-CSF, over
55 a time period of 5 min., similarly to published work [Kuwabara *et al.*, *Amer. J. Physiol. Endocrinol. Metabol.* 32: E1-E9 (1995)]. Briefly, 10⁶ cells were washed twice with PBS and then incubated on ice in labeled ligand for 30 min. to obtain surface complexes. The cells were again washed twice with ice-cold PBS and resuspended in MEM α at 37°C at t=0. The change in surface complexes and internal complexes was followed for a time period of 5 min.; a plot of internal

complexes versus the time integral of surface complexes yields a linear relationship, the slope of which is the complex internalization rate constant [Wiley *et al.*, *J. Biol. Chem.* 257: 4222-4229 (1982)]. No differences in internalization rate were detected between the wild-type G-CSF and the [His¹⁰⁹]G-CSF and [His¹¹²]G-CSF analogs. The errors associated with the internalization experiments are standard deviations of at least three independent experiments.

Recycling of G-CSF Analogs

[0183] Recycling experiments were performed on two of the G-CSF analogs, [His¹⁰⁹]G-CSF, [His¹¹²]G-CSF, identically to the internalization experiments above, except over a time period of 25 min.. Data from recycling experiments were parameter fitted to obtain recycling rate constants. The recycling rate constants for the mutants were found to be at least 50% greater than that of wild-type with 95% confidence by two-sample t test. The errors associated with the recycling experiments were standard deviations of at least three independent experiments. This improvement in ligand recycling was exactly the goal that was sought through the use of the computational modeling presented herein. While the measured increase in recycling is effectively from one round of internalization, the vast augmentation in mutant half-life results from the compounded effect of internalization, recycling, reinternalization and so on. Thus, the iterative effect of the recycling phenomenon can greatly improve drug potency by increasing its lifetime *in vivo* and reducing the negative feedback propagated by the drug-induced expansion of cells expressing the target receptor.

[0184] Accordingly, as indicated above, it has been found that the histidine substitutions of the present invention result in G-CSF analogs which have the same or greater potency relative to wild-type G-CSF in a cell proliferation assay. Also, the half-lives of the mutants were 6-10 times that of wild-type G-CSF. Furthermore, [His¹¹⁹]G-CSF induced cell proliferation to be almost twice that of wild-type G-CSF by day 8. In addition, ligand recycling was improved significantly, even though the internalization rate was unchanged. These results are significant, because they suggest that cellular trafficking of this mutant is improved relative to wild-type. Such changes in cellular response occur by affecting G-CSF receptor binding and/or the processes of sorting, recycling, and degradation via the ligand/receptor endocytic trafficking pathways.

[0185] The methodology presented herein is generalizable to other systems beyond the G-CSF/G-CSFR system as well. Given a crystal structure of a ligand-receptor complex, the "histidine switching" technique provides a framework for generating mutants with enhanced endosomal recycling of components of the trafficked complexes. This is the first work to demonstrate rational drug design in the context of a systems-level cellular trafficking analysis, rather than individual binding or signaling events *per se*.

[0186] While the present invention has been described in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations which come within the scope of the invention as claimed.

SEQUENCE LISTING

[0187]

<110> Sarkar, Casim
Lauffenburger, Douglas
Tidor, Bruce

<120> G-CSF Analog Compositions and Methods

<130> 01017/37377

<160> 8

<170> Patent In version 3.0

<210> 1
<211> 565
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (33)..(554)

EP 1 317 537 B1

<400> 1

	tctagaaaaa accaaggagg taataaataa tg	act cca tta ggt cct gct tct	53
5		Thr Pro Leu Gly Pro Ala Ser	
		1 5	
	tct ctg ccg caa agc ttt ctg ctg aaa tgt ctg gaa cag gtt cgt aaa		101
	Ser Leu Pro Gln Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg Lys		
	10	20	
10	atc cag ggt gac ggt gct gca ctg caa gaa aaa ctg tgc gct act tac		149
	Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu Lys Leu Cys Ala Thr Tyr		
	25	30	
	aaa ctg tgc cat ccg gaa gaa ctg gta ctg ctg ggt cat tct ctt ggg		197
15	Lys Leu Cys His Pro Glu Leu Val Leu Leu Gly His Ser Leu Gly		
	40	45	50
	atc ccg tgg gct ccg ctg tct tct tgc cca tct caa gct ctt cag ctg		245
	Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser Gln Ala Leu Gln Leu		
	60	65	70
20	gct ggt tgt ctg tct caa ctg cat tct ggt ctg ttc ctg tat cag ggt		293
	Ala Gly Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu Tyr Gln Gly		
	75	80	85
	ctt ctg caa gct ctg gaa ggt atc tct ccg gaa ctg ggt ccg act ctg		341
25	Leu Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu Gly Pro Thr Leu		
	90	95	100
	gac act ctg cag cta gat gta gct gac ttt gct act act att tgg caa		389
30	Asp Thr Leu Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Ile Trp Gln		
	105	110	115
	cag atg gaa gag ctc ggt atg gca cca gct ctg caa ccg act caa ggt		437
35	Gln Met Glu Glu Leu Gly Met Ala Pro Ala Leu Gln Pro Thr Gln Gly		
	120	125	130
			135
40			
45			
50			
55			

EP 1 317 537 B1

gct atg ccg gca ttc gct tct gca ttc cag cgt cgt gca gga ggt gta 485
Ala Met Pro Ala Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly Val
140 145 150

5 ctg gtt gct tct cat ctg caa tct ttc ctg gaa gta tct tac cgt gtt 533
Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr Arg Val
155 160 165

10 ctg cgt cat ctg gct cag ccg taatagaatt c 565
Leu Arg His Leu Ala Gln Pro
170

<210> 2
<211> 174
<212> PRT
15 <213> Homo sapiens

<400> 2
Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys
1 5 10 15

20 Cys Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln
20 25 30

25 Glu Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu Val
35 40 45

30 Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys
50 55 60

Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His Ser
65 70 75 80

35 Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser
85 90 95

40 Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala Asp
100 105 110

Phe Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro
115 120 125

45 Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala Phe
130 135 140

50 Gln Arg Arg Ala Gly Gly Val Leu Val Ala Ser His Leu Gln Ser Phe
145 150 155 160

Leu Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro
165 170

55 <210> 3
<211> 565

EP 1 317 537 B1

<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (33).. (554)

<220>
<221> misc_feature
<222> (359)
<223> y= c or t

<400> 3

15	tctagaaaaa accaaggagg taataaataa tg act cca tta ggt cct gct tct	53
	Thr Pro Leu Gly Pro Ala Ser	
	1 5	
20	tct ctg ccg caa agc ttt ctg ctg aaa tgt ctg gaa cag gtt cgt aaa	101
	Ser Leu Pro Gln Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg Lys	
	10 15 20	
25	atc cag ggt gac ggt gct gca ctg caa gaa aaa ctg tgc gct act tac	149
	Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu Lys Leu Cys Ala Thr Tyr	
	25 30 35	
30	aaa ctg tgc cat ccg gaa gaa ctg gta ctg gaa ggt cat tct ctt ggg	197
	Lys Leu Cys His Pro Glu Glu Leu Val Leu Glu Gly His Ser Leu Gly	
	40 45 50 55	
35	atc ccg tgg gct ccg ctg tct tct tgc cca tct caa gct ctt cag ctg	245
	Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser Gln Ala Leu Gln Leu	
	60 65 70	
40	gct ggt tgt ctg tct caa ctg cat tct ggt ctg ttc ctg tat cag ggt	293
	Ala Gly Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu Tyr Gln Gly	
	75 80 85	
45	ctt ctg caa gct ctg gaa ggt atc tct ccg gaa ctg ggt ccg act ctg	341
	Leu Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu Gly Pro Thr Leu	
	90 95 100	
50	gac act ctg cag cta cay gta gct gac ttt gct act act att tgg caa	389
	Asp Thr Leu Gln Leu His Val Ala Asp Phe Ala Thr Thr Ile Trp Gln	
	105 110 115	
55	cag atg gaa gag ctc ggt atg gca cca gct ctg caa ccg act caa ggt	437
	Gln Met Glu Glu Leu Gly Met Ala Pro Ala Leu Gln Pro Thr Gln Gly	
	120 125 130 135	
60	gct atg ccg gca ttc gct tct gca ttc cag cgt cgt gca gga ggt gta	485
	Ala Met Pro Ala Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly Val	
	140 145 150	
65	ctg gtt gct tct cat ctg caa tct ttc ctg gaa gta tct tac cgt gtt	533
	Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr Arg Val	
	155 160 165	

ctg cgt cat ctg gct cag ccg taatagaatt c
 Leu Arg His Leu Ala Gln Pro
 170

565

5

<210> 4

<211> 174

<212> PRT

10

<213> Homo sapiens

<400> 4

15

Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys
 1 5 10 15

Cys Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln
 20 25 30

20

Glu Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu Val
 35 40 45

25

Leu Glu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys
 50 55 60

30

Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His Ser
 65 70 75 80

Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser
 85 90 95

35

Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu His Val Ala Asp
 100 105 110

40

Phe Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro
 115 120 125

Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala Phe
 130 135 140

45

Gln Arg Arg Ala Gly Gly Val Leu Val Ala Ser His Leu Gln Ser Phe
 145 150 155 160

50

Leu Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro
 165 170

<210> 5

<211> 565

55

<212> DNA

<213> Homo sapiens

<220>

EP 1 317 537 B1

<221> CDS
<222> (33)..(554)

<220>
<221> misc_feature
<222> (368)
<223> y = c or t

<400> 5

10

tctagaaaaa accaaggagg taataaataa tg act cca tta ggt cct gct tct 53
Thr Pro Leu Gly Pro Ala Ser
1 5

15

tct ctg ccg caa agc ttt ctg ctg aaa tgt ctg gaa cag gtt cgt aaa 101
Ser Leu Pro Gln Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg Lys
10 15 20

20

atc cag ggt gac ggt gct gca ctg caa gaa aaa ctg tgc gct act tac 149
Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu Lys Leu Cys Ala Thr Tyr
25 30 35

25

aaa ctg tgc cat ccg gaa gct ctg gta ctg ctg ggt cat tct ctt ggg 197
Lys Leu Cys His Pro Glu Ala Leu Val Leu Leu Gly His Ser Leu Gly
40 45 50 55

atc ccg tgg gct ccg ctg tct tct tgc cca tct caa gct ctt cag ctg 245
Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser Gln Ala Leu Gln Leu
60 65 70

30

gct ggt tgt ctg tct caa ctg cat tct ggt ctg ttc ctg tat cag ggt 293
Ala Gly Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu Tyr Gln Gly
75 80 85

35

ctt ctg caa gct ctg gaa ggt atc tct ccg gaa ctg ggt ccg act ctg 341
Leu Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu Gly Pro Thr Leu
90 95 100

40

gac act ctg cag cta gat gta gct cay ttt gct act act att tgg caa 389
Asp Thr Leu Gln Leu Asp Val Ala His Phe Ala Thr Thr Ile Trp Gln
105 110 115

cag atg gaa gag ctc ggt atg gca cca gct ctg caa ccg act caa ggt 437
Gln Met Glu Glu Leu Gly Met Ala Pro Ala Leu Gln Pro Thr Gln Gly
120 125 130 135

45

gct atg ccg gca ttc gct tct gca ttc cag cgt cgt gca gga ggt gta 485
Ala Met Pro Ala Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly Val
140 145 150

50

ctg gtt gct tct cat ctg caa tct ttc ctg gaa gta tct tac cgt gtt 533
Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr Arg Val
155 160 165

55

ctg cgt cat ctg gct cag ccg taatagaatt c 565
Leu Arg His Leu Ala Gln Pro
170

<210> 6
<211> 174

EP 1 317 537 B1

<212> PRT
<213> Homo sapiens

<400> 6

5

Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys
1 5 10 15

10

Cys Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln
20 25 30

15

Glu Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Ala Leu Val
35 40 45

Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys
50 55 60

20

Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His Ser
65 70 75 80

25

Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser
85 90 95

Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala His
100 105 110

30

Phe Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro
115 120 125

35

Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala Phe
130 135 140

Gln Arg Arg Ala Gly Gly Val Leu Val Ala Ser His Leu Gln Ser Phe
145 150 155 160

40

Leu Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro
165 170

45

<210> 7
<211> 565
<212> DNA
<213> Homo sapiens

50

<220>
<221> misc_feature
<222> (389)
<223> y = c or t

55

<220>
<221> CDS
<222> (33).. (554)

EP 1 317 537 B1

<400> 7

5	tctagaaaaa accaaggagg taataaataa tg act cca tta ggt cct gct tct	53
	Thr Pro Leu Gly Pro Ala Ser	
	1 5	
10	tct ctg ccg caa agc ttt ctg ctg aaa tgt ctg gaa cag gtt cgt aaa	101
	Ser Leu Pro Gln Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg Lys	
	10 15 20	
15	atc cag ggt gac ggt gct gca ctg caa gct aaa ctg tgc gct act tac	149
	Ile Gln Gly Asp Gly Ala Ala Leu Gln Ala Lys Leu Cys Ala Thr Tyr	
	25 30 35	
20	aaa ctg tgc cat ccg gaa gaa ctg gta ctg ctg ggt cat tct ctt ggg	197
	Lys Leu Cys His Pro Glu Leu Val Leu Leu Gly His Ser Leu Gly	
	40 45 50 55	
25	atc ccg tgg gct ccg ctg tct tct tgc cca tct caa gct ctt cag ctg	245
	Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser Gln Ala Leu Gln Leu	
	60 65 70	
30	gct ggt tgt ctg tct caa ctg cat tct ggt ctg ttc ctg tat cag ggt	293
	Ala Gly Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu Tyr Gln Gly	
	75 80 85	
35	ctt ctg caa gct ctg gaa ggt atc tct ccg gaa ctg ggt ccg act ctg	341
	Leu Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu Gly Pro Thr Leu	
	90 95 100	
40	gac act ctg cag cta gat gta gct gac ttt gct act act att tgg cay	389
	Asp Thr Leu Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Ile Trp His	
	105 110 115	
45	cag atg gaa gag ctc ggt atg gca cca gct ctg caa ccg act caa ggt	437
	Gln Met Glu Glu Leu Gly Met Ala Pro Ala Leu Gln Pro Thr Gln Gly	
	120 125 130 135	
50	gct atg ccg gca ttc gct tct gca ttc cag cgt cgt gca gga ggt gta	485
	Ala Met Pro Ala Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly Val	
	140 145 150	
55	ctg gtt gct tct cat ctg caa tct ttc ctg gaa gta tct tac cgt gtt	533
	Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr Arg Val	
	155 160 165	
60	ctg cgt cat ctg gct cag ccg taatagaatt c	565
	Leu Arg His Leu Ala Gln Pro	
	170	
65	<210> 8	
	<211> 174	
	<212> PRT	
	<213> Homo sapiens	
70	<400> 8	

EP 1 317 537 B1

Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys
 1 5 10 15
 5 Cys Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln
 20 25 30
 10 Ala Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu Val
 35 40 45
 15 Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys
 50 55 60
 20 Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His Ser
 65 70 75 80
 25 Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser
 85 90 95
 30 Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala Asp
 100 105 110
 35 Phe Ala Thr Thr Ile Trp His Gln Met Glu Glu Leu Gly Met Ala Pro
 115 120 125
 40 Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala Phe
 130 135 140
 45 Gln Arg Arg Ala Gly Gly Val Leu Val Ala Ser His Leu Gln Ser Phe
 145 150 155 160
 50 Leu Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro
 165 170

Claims

1. A human Granulocyte Colony Stimulating Factor (G-CSF) analog polypeptide which comprises an amino acid substitution in the sequence of SEQ ID NO: 2 selected from the group consisting of:
 - a) a substitution of aspartic acid with histidine at position number 109, [His¹⁰⁹]G-CSF;
 - b) a substitution of aspartic acid with histidine at position number 112, [His¹¹²]G-CSF;
 - c) a substitution of glutamine with histidine at position number 119, [His¹¹⁹]G-CSF; and
 - d) any of the said analogs of subparts (a-c) optionally including an N-terminal methionyl residue.
2. A G-CSF analog polypeptide of claim 1 derivatized with one or more water soluble polymers.
3. A pharmaceutical composition comprising a G-CSF analog polypeptide according to claims 1 or 2, and a pharmaceutically acceptable carrier.

4. A polynucleotide encoding a human G-CSF analog polypeptide which comprises an amino acid substitution in the sequence of SEQ ID NO: 2 selected from the group consisting of:
 - a) a substitution of aspartic acid with histidine at position number 109, [His¹⁰⁹]G-CSF;
 - b) a substitution of aspartic acid with histidine at position number 112, [His¹¹²]G-CSF;
 - c) a substitution of glutamine with histidine at position number 119, [His¹¹⁹]G-CSF; and
 - d) any of the said analogs of subparts (a-c) optionally including an N-terminal methionyl residue.
5. A polynucleotide of claim 4 wherein said molecule is selected from the group consisting of:
 - a) the DNA sequences set forth in SEQ ID NOs: 3, 5, or 7 (and complementary strands); and
 - b) any of the DNA sequences of subpart (a) additionally encoding an N-terminal methionyl residue.
6. An expression construct containing a polynucleotide according to claim 4.
7. A host cell containing a polynucleotide according to claim 4.
8. The host cell of claim 7 wherein said host cell is selected from the group consisting of a bacterium, a mammalian cell, a cancer cell, a yeast cell, and an insect cell.
9. A process for producing G-CSF analog polypeptides [His¹⁰⁹]G-CSF, [His¹¹²]G-CSF, [His¹¹⁹]G-CSF, or the Mer¹ species thereof from a host cell containing nucleic acid encoding such analogs, wherein said process comprises:
 - (a) culturing said host cell containing a polypeptide according to claim 4 under conditions facilitating the expression of such polypeptide; and
 - (b) obtaining such G-CSF analog polypeptide.
10. Use of a polypeptide according to claims 1 or 2 for preparing a pharmaceutical composition for treating hematopoietic, neurological or reproduction related conditions.
11. The use of a polypeptide according to claim 10 wherein said condition is selected from the group consisting of: reduced hematopoietic function, reduced immune function, reduced neutrophil count, reduced neutrophil mobilization, mobilization of peripheral blood progenitor cells, sepsis, severe chronic neutropenia, bone marrow transplants, infectious diseases, leucopenia, thrombocytopenia, anemia, enhancing engraftment of bone marrow during transplantation, enhancing bone marrow recovery in treatment of radiation, chemical or chemotherapeutic induced bone marrow aplasia or myelosuppression, and acquired immune deficiency syndrome.
12. The use of a polypeptide according to claims 1 or 2 for the preparation of a pharmaceutical composition for sensitizing cells to chemotherapy and radiotherapy.
13. The use of a polypeptide according to any of claims 10 to 11 wherein said pharmaceutical composition includes at least one additional factor selected from among EPO, G-CSF, SCF, M-GDF, GM-CSF, M-CSF, CSF-1, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, interleukins, IGF-1, LIF, interferon, a neurotrophic factor, flt-3/flk-2 ligand, and a fibroblast growth factor.
14. A method for culturing hematopoietic cells *in vitro* comprising:
 - a) placing said cells in a suitable culture medium, said suitable culture medium containing a G-CSF analog polypeptide according to claim 1; and
 - b) providing suitable conditions for the growth of said hematopoietic cells.
15. The method of claim 14 wherein said culturing includes the use of at least one additional factor selected from among EPO, G-CSF, SCF, M-GDF, GM-CSF, M-CSF, CSF-1, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, interleukins, IGF-1, LIF, interferon, a neurotrophic factor, flt-3/flk-2 ligand, and a fibroblast growth factor.
16. A kit containing components for culturing hematopoietic cells comprised of:
 - a) any of the analog polypeptides of claim 1;

- b) components suitable for preparing medium for culturing hematopoietic cells; and,
c) optionally, at least one additional factor selected from among EPO, G-CSF, SCF, M-GDF, GM-CSF, M-CSF, CSF-1, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, interleukins, IGF-1, LIF, interferon, a neurotrophic factor, ftt-3/flk-2 ligand, and a fibroblast growth factor.

Patentansprüche

1. Zum menschlichen Granulozytenkolonie-stimulierenden Faktor (G-CSF) analoges Polypeptid, das in der Sequenz von SEQ ID NO: 2 eine Aminosäuresubstitution umfasst, ausgewählt aus der Gruppe bestehend aus:
 - a) Substitution von Asparaginsäure durch Histidin an Position Nummer 109, [His¹⁰⁹]G-CSF;
 - b) Substitution von Asparaginsäure durch Histidin an Position Nummer 112, [His¹¹²]G-CSF;
 - c) Substitution von Glutamin durch Histidin an Position Nummer 119, [His¹¹⁹]G-CSF; und
 - d) eines der Analoga der Unterteilungen (a-c), wahlweise einschließlich eines N-terminalen Methionylrestes.
2. Zu G-CSF analoges Polypeptid nach Anspruch 1, das mit einem oder mehreren wasserlöslichen Polymeren derivatisiert ist.
3. Pharmazeutische Zusammensetzung, umfassend ein zu G-CSF analoges Polypeptid gemäß den Ansprüchen 1 oder 2 und einen pharmazeutisch verträglichen Träger.
4. Polynukleotid, welches ein zum menschlichen G-CSF analoges Polypeptid kodiert, das in der Sequenz von SEQ ID NO: 2 eine Aminosäuresubstitution umfasst, ausgewählt aus der Gruppe, bestehend aus:
 - a) Substitution von Asparaginsäure durch Histidin an Position Nummer 109, [His¹⁰⁹]G-CSF;
 - b) Substitution von Asparaginsäure durch Histidin an Position Nummer 112, [His¹¹²]G-CSF;
 - c) Substitution von Glutamin durch Histidin an Position Nummer 119, [His¹¹⁹]G-CSF; und
 - d) eines der Analoga der Unterteilungen (a-c), wahlweise einschließlich eines N-terminalen Methionylrestes.
5. Polynukleotid nach Anspruch 4, wobei das Molekül aus der Gruppe, bestehend aus
 - a) den in SEQ ID NO: 3, 5, oder 7 (und den komplementären Strängen) dargelegten DNA-Sequenzen; und
 - b) einer der DNA-Sequenzen der Unterteilung (a), die zusätzlich einen N-terminalen Methionylrest kodiert,ausgewählt ist.
6. Expressionskonstrukt, das ein Polynukleotid gemäß Anspruch 4 enthält.
7. Wirtszelle, die ein Polynukleotid gemäß Anspruch 4 enthält.
8. Wirtszelle nach Anspruch 7, wobei die Wirtszelle aus der Gruppe, bestehend aus einem Bakterium, einer Säugierzelle, einer Krebszelle, einer Hefezelle und einer Insektenzelle, ausgewählt ist.
9. Verfahren zum Herstellen der zu G-CSF analogen Polypeptide [His¹⁰⁹]G-CSF, [His¹¹²]G-CSF, [His¹¹⁹]G-CSF oder deren Met¹-Spezies aus einer Wirtszelle, die Nukleinsäure enthält, welche derartige Analoga kodiert, wobei das Verfahren umfasst:
 - a) Kultivieren der Wirtszelle, die ein Polypeptid gemäß Anspruch 4 enthält, unter Bedingungen, die die Expression eines derartigen Polypeptids erleichtern; und
 - b) Erhalten derartiger zu G-CSF analoger Polypeptide.
10. Verwendung eines Polypeptids gemäß den Ansprüchen 1 oder 2 zum Herstellen einer pharmazeutischen Zusammensetzung zum Behandeln von hämatopoetischen, neurologischen oder mit der Fortpflanzung zusammenhängenden Leiden.
11. Verwendung eines Polypeptids gemäß Anspruch 10, wobei das Leiden aus der Gruppe, bestehend aus: reduzierter hämatopoetischer Funktion, reduzierter Immundefunktion, reduzierter Zahl neutrophiler Zellen, reduzierter Mobilisie-

5 rung neutrophiler Zellen, Mobilisierung peripherer Blutvorläuferzellen, Sepsis, schwerwiegende chronische Neutropenie, Knochenmarktransplantate, Infektionskrankheiten, Leukopenie, Thrombozytopenie, Anämie, Verbessern der Übertragung von Knochenmark während einer Transplantation, Verbessern der Knochenmarkerholung bei der Behandlung von strahlungs-, chemischer oder chemotherapeutisch induzierter Knochenmarkaplasie oder Myelosuppression, und erworbenem Immundefektsyndrom, ausgewählt ist.

12. Verwendung eines Polypeptids gemäß den Ansprüchen 1 oder 2 zur Herstellung einer pharmazeutischen Zusammensetzung zum Sensibilisieren von Zellen für Chemotherapie und Strahlentherapie.

10 13. Verwendung eines Polypeptids gemäß einem der Ansprüche 10 bis 11, wobei die pharmazeutische Zusammensetzung mindestens einen zusätzlichen Faktor, ausgewählt aus EPO, G-CSF, SCF, M-GDF, GM-CSF, M-CSF, CSF-1, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, Interleukinen, IGF-1, LIF, Interferon, einem neurotrophen Faktor, dem flt-3/flk-2-Liganden und einem Fibroblastenwachstumsfaktor, einschließt.

15 14. Verfahren zum Kultivieren hämatopoetischer Zellen *in vitro*, umfassend:

- a) Platzieren der Zellen in ein geeignetes Kulturmedium, wobei das geeignete Kulturmedium ein zu G-CSF analoges Polypeptid gemäß Anspruch 1 enthält; und
- b) Bereitstellen geeigneter Bedingungen für das Wachstum der hämatopoetischen Zellen.

20 15. Verfahren nach Anspruch 14, wobei das Kultivieren die Verwendung von mindestens einem zusätzlichen Faktor, ausgewählt aus EPO, G-CSF, SCF, M-GDF, GM-CSF, M-CSF, CSF-1, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, Interleukinen, IGF-1, LIF, Interferon, einem neurotrophen Faktor, dem flt-3/flk-2-Liganden und einem Fibroblastenwachstumsfaktor, einschließt.

25 16. Kit, das Komponenten zum Kultivieren hämatopoetischer Zellen enthält, bestehend aus:

- a) einem der analogen Polypeptide nach Anspruch 1;
- b) Komponenten, die zum Herstellen von Medium zum Kultivieren hämatopoetischer Zellen geeignet sind; und
- c) wahlweise mindestens einem zusätzlichen Faktor, ausgewählt aus EPO, G-CSF, SCF, M-GDF, GM-CSF, M-CSF, CSF-1, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, Interleukinen, IGF-1, LIF, Interferon, einem neurotrophen Faktor, dem flt-3/flk-2-Liganden und einem Fibroblastenwachstumsfaktor.

35 Revendications

1. Polypeptide analogue du facteur de croissance des colonies granulocytaires humain (G-CSF) qui comprend une substitution d'acide aminé dans la séquence de SEQ ID NO:2, choisie dans le groupe constitué par :

- a) une substitution de l'acide aspartique par l'histidine à la position numéro 109, [His¹⁰⁹]G-CSF ;
- b) une substitution de l'acide aspartique par l'histidine à la position numéro 112, [His¹¹²]G-CSF ;
- c) une substitution de la glutamine par l'histidine à la position numéro 119, [His¹¹⁹]G-CSF ; et
- d) l'un quelconque desdits analogues des sous-paragraphe (a-c) contenant éventuellement un résidu méthionyle N-terminal.

2. Polypeptide analogue du G-CSF selon la revendication 1, dérivatisé avec un ou plusieurs polymères solubles dans l'eau.

3. Composition pharmaceutique comprenant un polypeptide analogue du G-CSF selon la revendication 1 ou 2, et un véhicule pharmaceutiquement acceptable.

4. Polynucléotide codant un polypeptide analogue du G-CSF humain qui comprend une substitution d'acide aminé dans la séquence de SEQ ID NO:2, choisie dans le groupe constitué par :

- a) une substitution de l'acide aspartique par l'histidine à la position numéro 109, [His¹⁰⁹]G-CSF ;
- b) une substitution de l'acide aspartique par l'histidine à la position numéro 112, [His¹¹²]G-CSF ;
- c) une substitution de la glutamine par l'histidine à la position numéro 119, [His¹¹⁹]G-CSF ; et
- d) l'un quelconque desdits analogues des sous-paragraphe (a-c) contenant éventuellement un résidu méthio-

nyle N-terminal.

5. Polypeptide selon la revendication 4, dans lequel ladite molécule est choisie dans le groupe constitué par :

- a) les séquences d'ADN présentées dans les SEQ ID NOs: 3, 5 ou 7 (et les brins complémentaires) ; et
- b) l'une quelconque des séquences d'ADN du sous-paragraphe (a) codant de plus un résidu méthionyle N-terminal.

6. Construction d'expression contenant un polynucléotide selon la revendication 4.

7. Cellule hôte contenant un polynucléotide selon la revendication 4.

8. Cellule hôte selon la revendication 7, dans laquelle ladite cellule hôte est choisie dans le groupe constitué par une bactérie, une cellule de mammifère, une cellule cancéreuse, une cellule de levure, et une cellule d'insecte.

9. Procédé pour produire des polypeptides analogues du G-CSF [His¹⁰⁹] G-CSF, [His¹¹²]G-CSF, [His¹¹⁹]G-CSF, ou les espèces Met⁻¹ de ceux-ci, à partir d'une cellule hôte contenant un acide nucléique codant de tels analogues, dans lequel le procédé comprend :

- (a) la culture de ladite cellule hôte contenant un polypeptide selon la revendication 4 dans des conditions facilitant l'expression d'un tel polypeptide ; et
- (b) l'obtention d'un tel polypeptide analogue du G-CSF.

10. Utilisation d'un polypeptide selon la revendication 1 ou 2 pour la préparation d'une composition pharmaceutique destinée au traitement d'états hématopoïétiques, neurologiques, ou liés à la reproduction.

11. Utilisation d'un polypeptide selon la revendication 10, dans laquelle ledit état est choisi dans le groupe constitué par une fonction hématopoïétique réduite, une fonction immunitaire réduite, un compte de neutrophiles réduit, une mobilisation de neutrophiles réduite, une mobilisation de cellules souches du sang périphérique, une sepsie, une neutropénie chronique sévère, des transplantations de moelle osseuse, des maladies infectieuses, une leucopénie, une thrombocytopénie, une anémie, une amplification du greffage de moelle osseuse durant une transplantation, une amplification de la récupération de moelle osseuse dans le traitement d'une myélodépresseion ou d'une aplasie de moelle osseuse induite par voie chimique ou chimiothérapeutique ou par irradiation, et un syndrome d'immuno-déficience acquise.

12. Utilisation d'un polypeptide selon la revendication 1 ou 2, pour la préparation d'une composition pharmaceutique destinée à sensibiliser des cellules à une chimiothérapie et une radiothérapie.

13. Utilisation d'un polypeptide selon l'une quelconque des revendications 10 et 11, dans laquelle ladite composition pharmaceutique contient au moins un facteur additionnel choisi parmi l'EPO, le G-CSF, le SCF, le M-GDF, le GM-CSF, le M-CSF, le CSF-1, l'IL-1, l'IL-2, l'IL-3, l'IL-4, l'IL-5, l'IL-6, l'IL-7, l'IL-8, l'IL-9, l'IL-10, l'IL-11, l'IL-12, les interleukines, l'IGF-1, le LIF, l'interféron, un facteur neurotrophique, le ligand fit-3/flk-2, et un facteur de croissance des fibroblastes.

14. Procédé pour cultiver des cellules hématopoïétiques *in vitro* comprenant :

- a) le placement desdites cellules dans un milieu de culture convenable, ledit milieu de culture convenable contenant un polypeptide analogue du G-CSF selon la revendication 1 ; et
- b) la fourniture de conditions convenables pour la croissance desdites cellules hématopoïétiques.

15. Procédé selon la revendication 14, dans lequel ladite culture comprend l'utilisation d'au moins un facteur additionnel choisi parmi l'EPO, le G-CSF, le SCF, le M-GDF, le GM-CSF, le M-CSF, le CSF-1, l'IL-1, l'IL-2, l'IL-3, l'IL-4, l'IL-5, l'IL-6, l'IL-7, l'IL-8, l'IL-9, l'IL-10, l'IL-11, l'IL-12, les interleukines, l'IGF-1, le LIF, l'interféron, un facteur neurotrophique, le ligand fit-3/flk-2, et un facteur de croissance des fibroblastes.

16. Trousse contenant des composants pour la culture de cellules hématopoïétiques, constituée par :

- a) l'un quelconque des polypeptides analogues de la revendication 1 ;

EP 1 317 537 B1

b) des composants convenables pour la préparation d'un milieu destiné à la culture de cellules hématopoïétiques ; et

c) éventuellement au moins un facteur additionnel choisi parmi l'EPO, le G-CSF, le SCF, le M-GDF, le GM-CSF, le M-CSF, le CSF-1, l'IL-1, l'IL-2, l'IL-3, l'IL-4, l'IL-5, l'IL-6, l'IL-7, l'IL-8, l'IL-9, l'IL-10, l'IL-11, l'IL-12, les interleukines, l'IGF-1, le LIF, l'interféron, un facteur neurotrophique, le ligand flt-3/flk-2, et un facteur de croissance des fibroblastes.